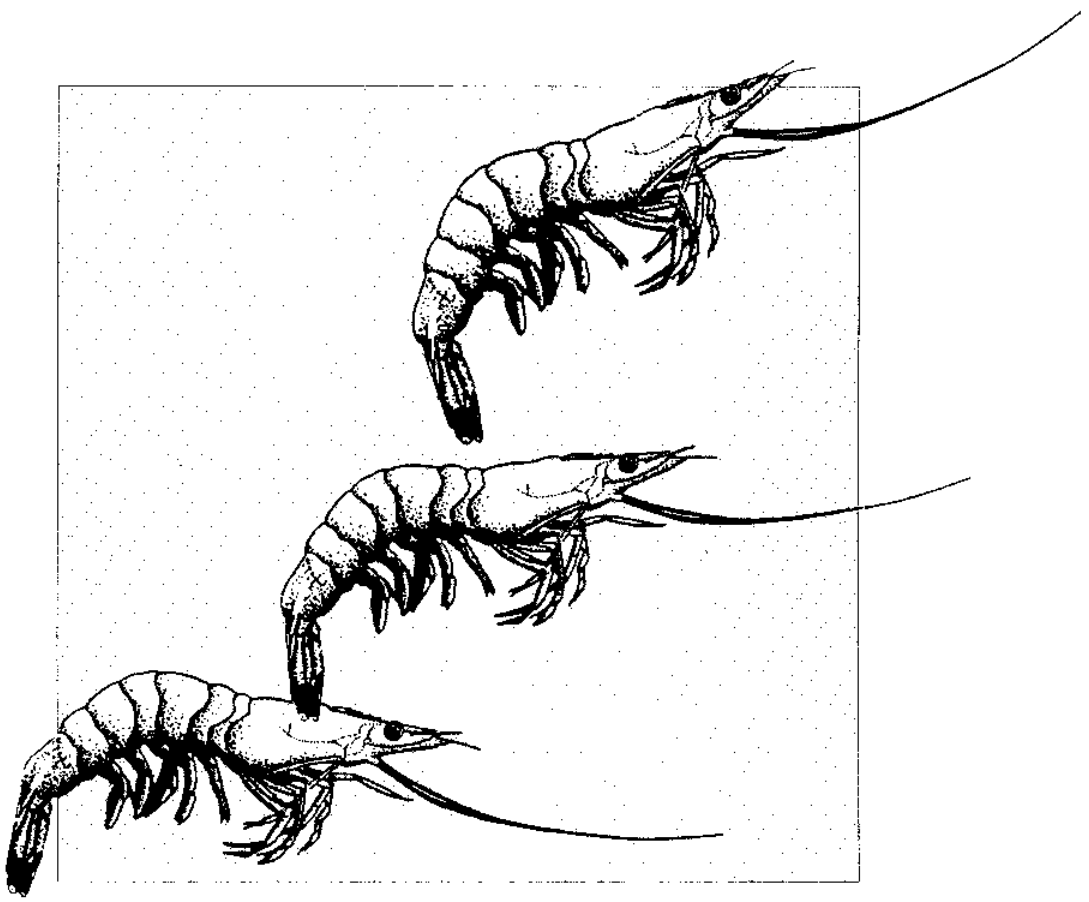
LABORATORY MANUAL FOR THE CULTURE OF

PENAEID SHRIMP LARVAE



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MARINE ADVISORY SERVICE
SEA GRANT COLLEGE PROGRAM
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Laboratory Manual for the Culture of Penaeid Shrimp Larvae

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I. INTRODUCTION

This manual does not purport to be a complete text on shrimp culture, nor even on larval culture. It is, rather, designed as an aid to a short general introductory laboratory course in shrimp culture. To this end, several sources were used, in some cases almost *in tota*. We wish to extend our sincere thanks to all those who, wittingly or unwittingly, helped us. A bibliography is included herewith and acknowledgments are made at the end of the manual.

In worldwide commercial shrimp aquaculture, penaeids are primarily cultured. These species include *monodon, japonicus, vannamei, stylirostris* and others. Because *P. vannamei* is, at the moment, the most popularly cultured species in the Western Hemisphere and is the most available to us, we have chosen to concentrate on its culture in this laboratory manual. However, for the most part, the general techniques presented here can be used in the cultivation of any of the above species. Where applicable, the differences between the specific species will be mentioned.

To learn the conditions required for the survival of shrimp, zoologists have studied the natural environmental conditions of the various stages of metamorphosis and maturation. Hence, they have learned to raise shrimp through all phases of their development, including: maturation, spawning, hatching, larval rearing and grow-out. (See Figures 1 and 2, Page 2). It is these ideal natural environmental conditions which we attempt to emulate in the laboratory and commercial facility.

Figure 1
Major Aspects of Shrimp Mariculture

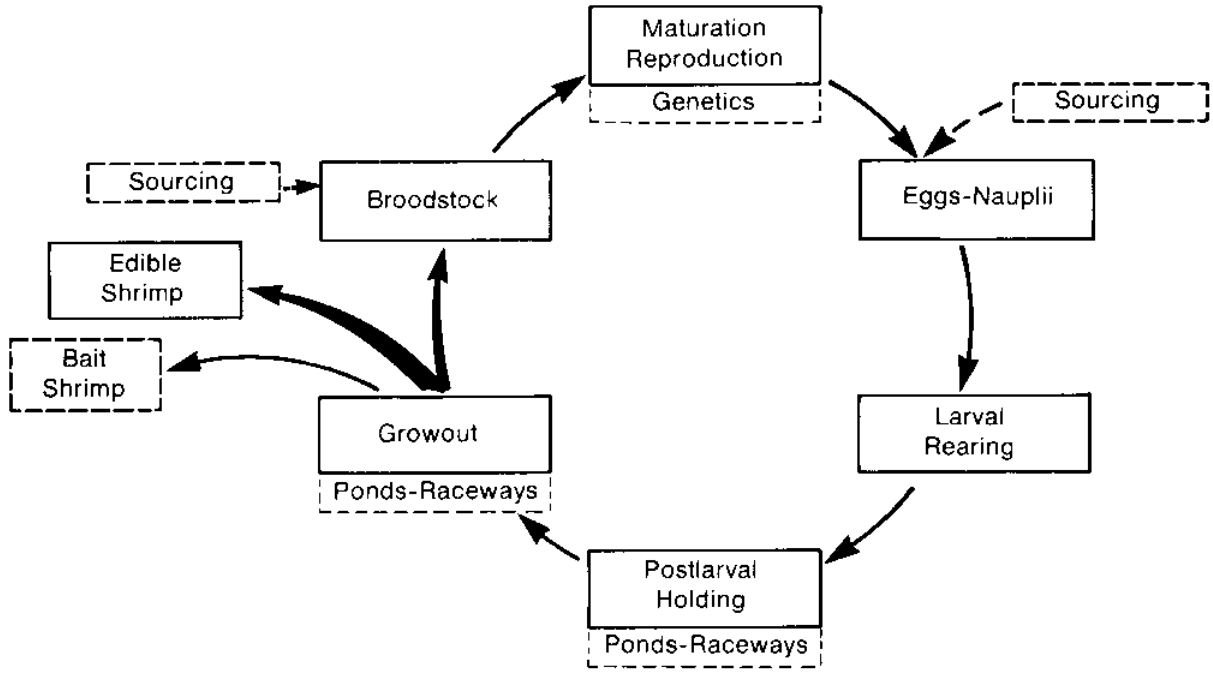
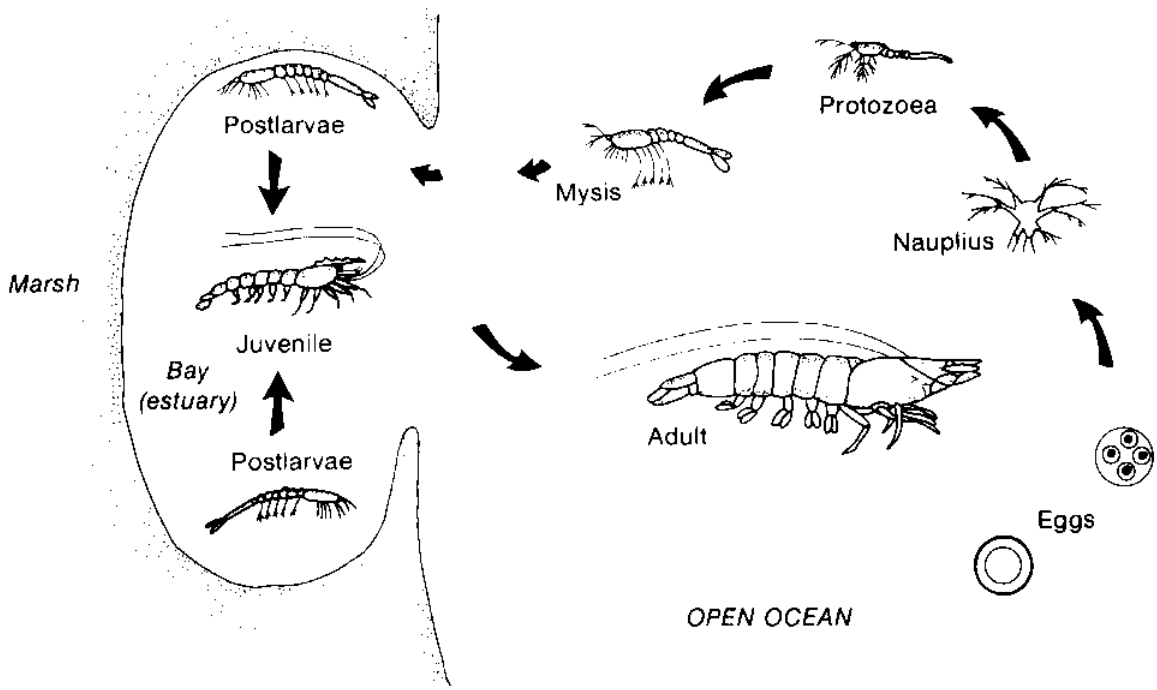


Figure 2
Penaeid Shrimp Life Cycle



II. INTRODUCTION TO THE LABORATORY

During this course, we will spend a considerable amount of time in the laboratory. Each participant will get hands-on larval rearing experience. You will be asked to work together in groups, each group being allocated a larval rearing tank.

It is intended that each group will stock their own larvae and raise them through their "most critical" stages of metamorphosis. This will require daily inspection and recording of their stage and condition. The water quality (including temperature, salinity, pH and contamination) and availability of food (first algae, then *Artemia*) will be monitored, recorded and controlled. We will all participate in raising algae and *Artemia* (brine shrimp) and preparing them for use. Each team will then ascertain the requirements of their larvae and feed them appropriately.

We will become acquainted with and make use of the instruments, equipment and procedures most often used in larval rearing facilities. Most of these will be covered in this laboratory manual.

Wet Laboratory

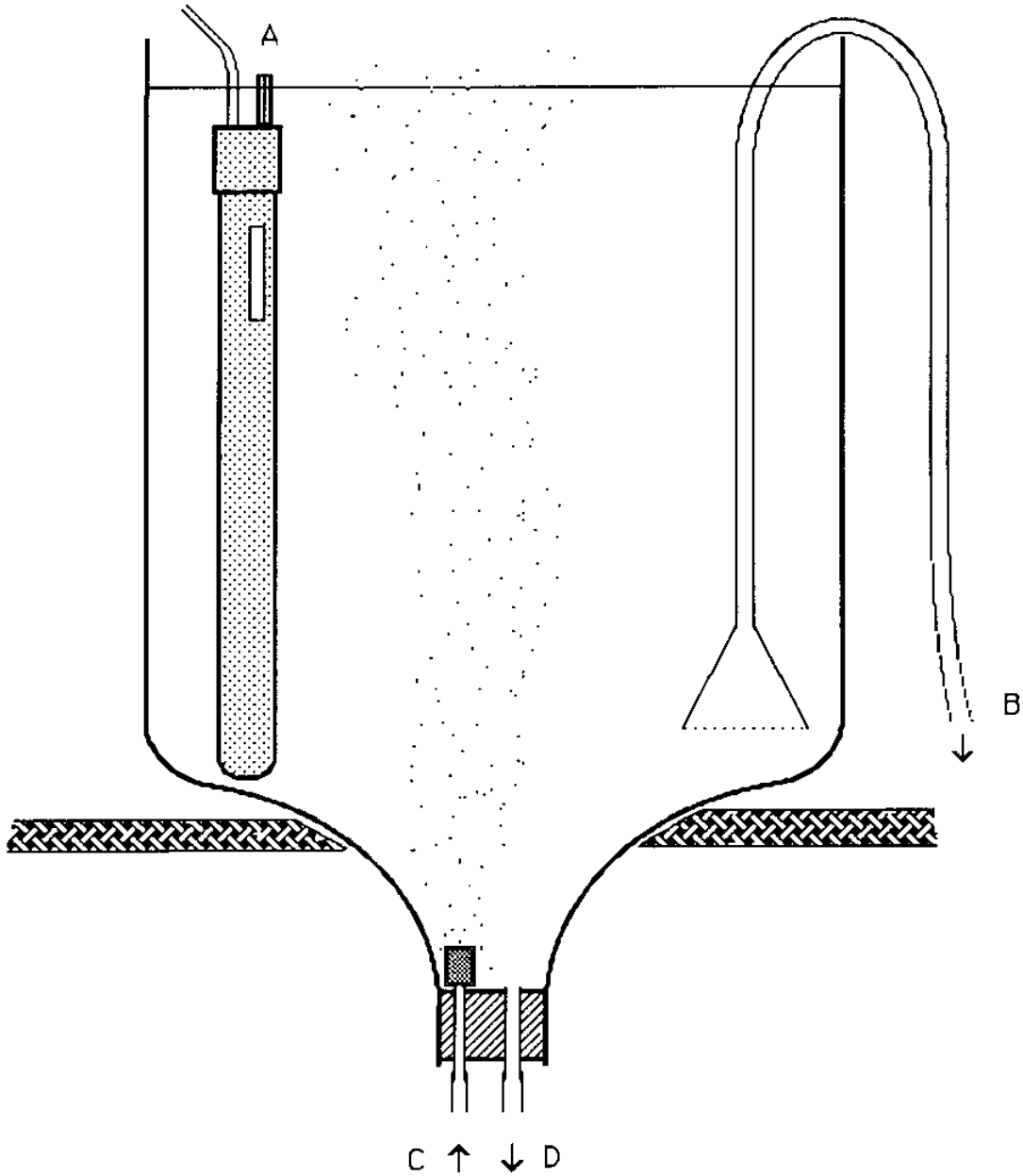
SYSTEMS:

Larval Rearing Tanks (LRT): The LRTs consist of a rack of 12- by 16-liter (5 gallon), conical-bottomed carboys. They are of clear plastic and are open topped to afford inspection and allow handling. The stopper in the bottom has two penetrations. One is for drainage and the other, for aeration (see Figure 3, Page 4).

Seawater: Seawater, with a salinity between 28 to 36 ppt. (parts per thousand), is held in settling tanks. Here, the coarser suspended particles are allowed to precipitate out before the water is filtered and pumped into a reservoir. In the laboratory we pass it through a 10- and a 1-micron cotton cartridge filter to remove fine particles and finally an ultraviolet filter to sterilize it. New water is allowed to recirculate through the filter system continuously until it is used. Chlorination of the seawater may be necessary, depending upon the location of the laboratory.

The drain in the tank stoppers should **not** be used, as larvae could themselves be lost. Rather, when exchanging water, a siphon with a Nitex® filter is used to drain the medium, while excluding the larvae. The Nitex® screen should be just fine enough to exclude the larvae (this will range from 100 µm, at stage N1, to 300 µm, at the mysis stage).

Electricity: Electricity is delivered by a socketed strip, mounted above the tanks.



A THERMOSTATICALLY CONTROLLED HEATER
B SIPHON, WITH MESH SIEVE
C AERATION WITH AIRSTONE
D DRAINAGE PIPE; DO NOT USE

FIGURE 3: LARVAL REARING TANK

WARNING: PLEASE BE SURE THAT HANDS, PLUGS, ETC., ARE DRY BEFORE WORKING WITH THE ELECTRICAL EQUIPMENT.

Lighting: Lighting is supplied continuously by two neon tubes, at an intensity of 950 to 1,200 LUX at the medium surface.

Temperature: The temperature of the medium is maintained at approximately 28°C, by thermostatically controlled immersible heaters. The "knob" at the top of the heater allows adjustment of the thermostat (clockwise rotation will increase temperature, while counter-clockwise rotation will decrease it). This should be done initially by immersing the heater in 28°C water. The thermostat is then adjusted until the light just flickers on and off. It should then be monitored constantly for 15 minutes after and intermittently for two hours thereafter. Should the temperature be more than 1°C above or below 28°C, a *very slight* adjustment should be made, followed by careful monitoring. The thermostats are very volatile and lack of patience can quickly result in "boiled" shrimp.

NOTE: WHEN NOT FULLY IMMERSED IN LIQUID, THE HEATERS WILL BURN OUT. THEY SHOULD BE UNPLUGGED BEFORE THEIR REMOVAL FROM THE TANKS.

Air: Air is supplied by a compressor, located above the LRT rack, via PVC piping and a PVC needle valve. Clear plastic (Tygon®) tubing is used between the valve and the airstone.

The aeration should be set at a slow, steady, stream of bubbles. The aeration should be strong enough to facilitate a gentle circulation in the tank, but not strong enough to batter the larvae or to prevent them from feeding. When inspecting the larvae, fold or pinch the plastic tubing, rather than shut off the valve.

INSTRUMENTS: The instruments which we will use in the lab are primarily water quality measuring devices. They include: the thermometer, the pH meter (for measuring acidity/alkalinity) and the refractometer (for measuring salinity). Further, we will be introduced to the chemical analysis kit (with this we run various titrations for ammonia, nitrates, etc.).

Dry Laboratory

SYSTEMS: The only additional supply which we will use here is distilled water.

INSTRUMENTS: In the dry lab, we will use both dissecting and compound microscopes, the hemacytometer (for counting algal cells) and various other general lab instruments. Basic information on the use of the major instruments can be found in the Technical Sheets beginning on Page 68.

RECORDS: The systematic maintenance of records can be a useful aid in larval care. They serve as a means of communication between the hatchery team members. When several tanks are to be tended, late at night, it is easy to forget the various measurements and even entire steps in the process. Well formulated tables for record keeping can minimize mistakes and can also aid in training the new team members.

Records can be used, not just for day-to-day planning, but also for "fine-tuning", ordering supplies, long-term planning and expansion. They can also be used to pin-point a problem or potential problem. One way to keep records, which has proved to be useful in a commercial operation, is by recording the collected daily data on a "Larval Rearing Tank Data Log" (see Figure 4, Page 7). Another important log is the "Maturation/Hatchery Egg-Nauplii Data Log" (see Figure 5, Page 8). The "Larval - State of Health" form (see Figure 6, Page 9) provides a format for the subjective grading of new batches of larvae. By grading each new batch for various criteria, it is used to build an overall numerical score for that batch. The form can be used to train new hatchery personnel in evaluation techniques. Afterwards, its use can be discontinued, but the principles are still followed when evaluating a batch.

Figure 4

Larval Rearing Tank Data Log

Nauplii Score: _____
LRT Number: _____
LRT Volume: _____
Nauplii Stocked: _____
P¹ Pop. Estimate: _____

Batch Number: _____
PLs. Harvested: _____
% Survival N-PL: _____
% Survival P¹1-PL: _____

Date	Time	Stage	Health State	Temp	pH	Food Type	Cell Count Before	Cell Count After	Artemia Counts/data			Comments
									#Art./ml	#Desired	#Art. Fed	

Figure 5

Maturation/Hatchery
Egg-Nauplii Data Log

Date	Time	Stage	Source St. or Mat.	#Source	# Total Egg Nauplii	# Fertile Eggs	Batch#	Transferred to LRT#

Comments

Figure 6

Larval-State of Health

Batch # _____ Date _____

Time _____

Name _____

Microscopic Observations	Obs.:	Activity	General Appearance	Absence of Deformities	Absence of Mucus External debris-bacteria	Setae Developed	Absence of Bent Setae	Photo-Positive Reaction	# Dead	Comments
Larval Sample	#1									
	#2									
	#3									
	#4									
	#5									
	#6									
	#7									
	#8									
	#9									
	#10									
Total Average										Score

Score Ratings:
#3. Excellent, Highest, Most.
#2. Fair, Mid-Range, Average.
#1. Poor, Lowest, Least.

III. LARVAL CULTURE

Introduction

In their natural environment, adult shrimp make their way offshore to clean, stable, oceanic water (see Figure 2, Page 2). Here they mature, mate and the females spawn their eggs. The eggs will sink, but within about 14 hours the eggs hatch and the nauplii, being strongly phototropic, swim towards the surface.

These larvae will pass through three distinct stages, naupliar, protozoeal (or zoeal) and mysis, before metamorphosing into postlarval shrimp. Their diet ranges from the hereditary yolk sack, during the early naupliar stage, to phytoplankton (microscopic plant organisms) and then to zooplankton (microscopic animals). Finally, at mysis stage and beyond, the shrimp is able to eat a wide variety of organisms, including *Artemia* (brine shrimp).

During this period, the larvae drift with the currents. A small percent of them are swept into the bays and estuaries by the currents. Here, the postlarvae remain, through their juvenile months, until they too mature and seek the offshore spawning grounds (see Figure 2, Page 2). It has been estimated that only 1 percent of those spawned in nature actually reach the adult stage.

LARVAL STAGES AND THEIR IDENTIFICATION (Yang, 1975): It is important that the aquaculturist be able to ascertain not only the major stages of metamorphosis, but also the substages. This allows the requirements for food, tanks, etc. to be anticipated accurately and for the correct rate of feeding to be made.

Naupliar Stage: The Indo-Pacific species (*monodon, indicus* and *japonicus*) have 6 naupliar substages, whereas the New World species (*vannamei* and *stylirostris*) have only 5 substages. A general pattern of 3 substages for each of the zoeal and mysis stages is universally reported, with only a few exceptions. The duration of each substage varies with the species and rearing temperature.

Data on the larval development of Penaeid shrimp is presented in Table I, Pages 13 and 14. For each substage, this includes the approximate duration of the substage, the size of the larvae and their required food and environmental conditions. Additional comments, of value to the culturist, are also included.

The nauplii hatch from the eggs in a folded-over position, but quickly straighten out. After several minutes they begin to swim, slowly at first, but within half an hour, more briskly. Swimming is accomplished by movement of the three pairs of appendages in paddle-like fashion, which produces a zig-zag roll of the body (Hudinaga, 1942). They

swim briefly and then rest. The nauplii are strongly phototropic and swim in the direction of the light source. A rapid response by the nauplii to a light source indicates that they are in good health. When at rest, the nauplii are suspended in a somewhat perpendicular position with the dorsal side of the body downward and the appendages slanted upward. During the last naupliar substage, the body becomes somewhat flattened.

Figure 7, Page 15, illustrates the substages of the shrimp larval stages, emphasizing those characteristics which can be used for identification. More detailed characteristics of the five naupliar substages of the species *P. duorarum* are shown in Figure 8, Page 16. Characteristics used in the identification of the individual substages are listed in the legend and indicated on the figure by arrows.

Protozoeal Stage: In the zoeal stages, swimming is accomplished with the first and second antennae, as in the naupliar stage, but these are now aided by the well-developed first and second maxillipeds. The swimming stroke is slower than that of the nauplii, the movement appears less jerky. Characteristic of the zoeae is their continuous feeding. The culturist can judge how well the zoeae are feeding by the contractions of the digestive tract and the presence of long trail of feces. Active feeding and a continued prompt response to a light source are indications of healthy zoeae. Towards the end of the last zoeal substage, the body becomes slightly flexed.

Detailed characteristics of the 3 zoeal substages of *P. duorarum* are shown in Figure 9, Page 17 (from Dobkin, 1961). As in the illustration of the nauplii, characteristics used in identifications of the zoeal substages are indicated by arrows. The size ranges given in the diagnoses below are based on measurements of *P. japonicus*.

Z1: 1. Body length 0.86 - 1.32 mm.
2. Body flattened; carapace distinct.
3. Sessile eyes present.
4. First and second maxillae and first and second maxillipeds functional.
5. Furcal processes present.
6. Digestive tract visible.

Z2: 1. Body length 1.33 - 2.13 mm.
2. Stalked eyes present.
3. Rostrum developed.
4. Supraorbital spines developed.
5. Abdominal segmentation apparent.

Z3: 1. Body length 2.14 - 2.70 mm.
2. Abdominal segmentation distinct; dorsal and/or lateral spines present on most somites.
3. Rudiments of uropods present.

Mysis Stage: In the mysis stage, the antennae are reduced and swimming becomes a function of the pereiopods, with some assistance from the three pairs of maxillipeds. In swimming the mysis body is flexed, with the head lowered; movement is in a backward direction. In this stage, there is less tendency for the myses to be attracted to light.

Detailed characteristics of the three myses substages are shown in Figure 10, Page 18 (from Dobkin, 1961), and are based on *P. duorarum*. As in the illustration of the nauplii, characteristics used in identifications of the mysis substages are indicated by arrows. The size ranges given in the diagnoses below are based on measurements of *P. japonicus*.

M1: 1. Body length 2.67 - 3.40 mm.

2. Body shrimp-like in shape.

3. Pereiopods well developed.

4. First and second antennae reduced.

5. Uropods well developed.

6. Primordial pleopod buds present.

M2: 1. Body length 2.99 - 3.90 mm.

2. Unsegmented pleopod buds present.

M3: 1. Body length 3.70 - 4.52 mm.

2. Pleopods developed, segmented.

POSTLARVAE: During the first 4 or 5 days of postlarval life, the animals are planktonic. In subsequent stages, they can be seen to cling to the walls of the tank or will take up a completely demersal life. By substage Pl_7 (7-day-old postlarvae), the larvae of burrowing species often are able to burrow in the sand. Feeding by the postlarvae is accomplished by the chelate periopods, which are able to grasp and hold food. Pleopods are used in swimming.

Detailed characteristics of the first Pl substage are shown in Figure 10, Page 18. The size ranges given in the diagnoses, on this page, are based on measurements of *P. japonicus* and *P. duorarum*. As in the illustration of the nauplii, characteristics used in identifications of the mysis substages are indicated by arrows.

Pl_1: 1. Body length 4.79 - 5.00 mm.

2. First three pairs of pereiopods chelated.

3. Pleopods with setae.

Table I

SHRIMP LARVAE DATA

(from Treece, 1985)

	Form or Stage and Various Names	Time in Stage	Size at End of Stage *	Common Food	Parameters (opt.)	Additional Comments and Observations
1	Egg	Approx. 14 hrs @ 28°C	Approx. 220 Microns in dia. (0.22mm)		Water as clean as possible. Surface area of container important as little aeration as necessary to maintain high D.O. (5ppm+), but prevent abrasion of egg membrane. pH 8.0. Eggs sink and should not be overcrowded (Layered). Temp. 28°C (opt)	Eggs are flexible, elastic and can be forced through a 202 micron screen mesh if under any type of water pressure. To eliminate the possibility of eggs passing- A #155 nitex screen is used.
2	Nauplius (N) (plural Nauplii) NI, NII, NIII, NIV, NV	48 hrs @28°C (Range: 36-51 hrs)	NV Mean Total Length = 0.50 mm Range (0.43-0.58mm). Mean Width = 0.20mm, Range (0.18-0.22mm)	Yolk	Water clean as possible pH 8.0. Gentle aeration to maintain high D.O. (5ppm+) or water current to keep them suspended;they will surface otherwise. Photopositive at this stage. Temp. 28°C	Swim only occasionally but with more frequency as they develop. (Example: Stage NI may swim 3 strokes, lie dormant for 30 seconds, then swim 3 strokes, lie dormant 5 sec. then swim.) (#155 Nitex)
3	Protozoea I (PI) (P^1) also referred to as Zoea I or ZI.	40 hrs. Average (Range: 36-48 hrs. @28°C	Mean Total Length 1.0 mm Mean Caudal (tail) Length 0.3 mm	Phyto-plankton 3-5μ in size	(Minimum algal cell count which animals are exposed to is 1 x 10^5 algal cells/ml.) pH 8.0 (opt.) Temp. 28°C Ammonia - Less than 25 micro gram atoms/liter	Continuously swimming and consuming phyto-plankton. Fecal threads visible and are often longer than animal. #202 Nitex used to retain animals
4	Protozoea II (PII) (P^2) also called Zoea II or ZII	40 hrs. Average (Range 36-48 hrs.) @28°C	Mean Total Length 1.71 mm (Range 1.28-2.01 mm). Mean Caudal (tail) Length 0.80 mm (Range 0.72-0.87 mm)	" 5-10μ in size.	"	Presence of stalked-compound eyes #202 Nitex used to retain animals

	Form or Stage and Various Names	Time in Stage	Size at End of Stage *	Common Food	Parameters (opt.)	Additional Comments and Observations
5	Protozoea III (PIII) (P^3)(also called Zoea III or ZIII)	40 hrs. Average (28°C) Range 36-48 hrs.	Mean Total Length 2.59 mm (2.4-2.59mm Range) Mean Caudal (tail) Length 1.06 mm (Range 0.93-1.40 mm)	Phyto-plank-ton Mixed Species	pH 8.0 (opt.) same as PI & PII Ammonia - Less than 25 µg at/l Temp. 28°C	Biramous uropods and spines on abdominal segments #202 Nitex used to retain animals.
6	Mysis I (MI) (M_1)	24 hrs. (28°C)	Mean Total Length 3.5 mm Mean Caudal (tail) Length 1.2 mm	*Artemia* and phyto-plank-ton	Fed the combination of phytoplankton and *Artemia*. pH 8.0 Same parameters as protozoea. Temp. 28°C Ammonia - Less than 25 µg at/l	Pleopods small just beginning to protrude from ventral side of abdominal segments. Animals able to flip backwards as well as swim forward. #202 Nitex effluent screen used to retain *Artemia* nauplii.
7	Mysis II (MII) (M_2)	24 hrs.	Mean Total Length 3.8 mm (Range 3.3-4.2 mm) Mean Caudal (tail) Length 1.3 mm (Range 1.2-1.4 mm)	*Artemia* and phyto-plank-ton	pH 8.0 Temp. 28°C Ammonia - Less than 25 µg at/l.	Unsegmented pleopods. but more pronounced and curving to anterior. #202 Nitex used to retain *Artemia* nauplii.
8	Mysis III MIII (M_3)	24 hrs.	Mean Total Length 4.3 mm (Range 3.9-4.7 mm) Mean Caudal (tail) Length 1.4 mm (Range 1.3-1.5 mm)	*Artemia* and phyto-plank-ton	pH 8.0 Temp. 28°C Ammonia - Less than 25 µg at/l.	Pleopods composed of 2 segments and 2 - 3 terminal setae. #202 Nitex to retain *Artemia*
9	Post larva I (PI I) (PL_1)	24 hrs.	Mean Total Length 4.6 mm (Range 4.2-5.0 mm) Mean Caudal (tail) Length 1.5 mm (Range 1.4-1.6 mm)	*Artemia* and phyto-plank-ton	pH 8.0 Temp. 28°C Ammonia - Less than 25 µg at/l.	Generally - Post-larvae spend approx. 6 days suspended in water column but prefer to be bottom dwellers thereafter. #202 - to retain *Artemia* (food supply) (#500 Nitex to retain animals).

* These sizes are based on measurements using *P. aztecus*. *P. sty.* and *P. vann* are slightly larger (example: we have measured a number of *P. sty.*, PL_1's at 6 mm total length.)

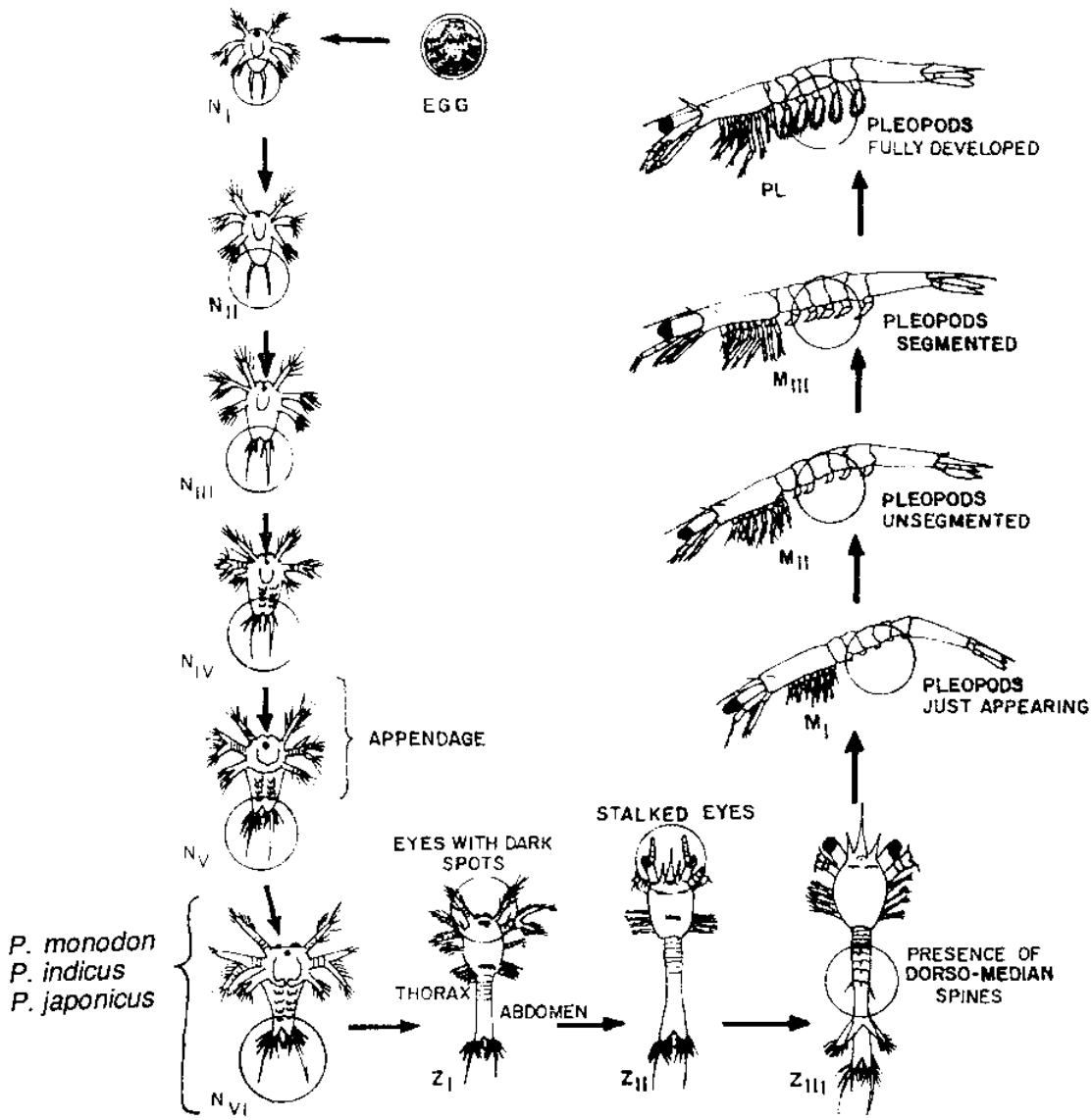


Figure 7 Shrimp Larval Stages
(after Motoh, 1979)

Figure 8 Naupliar substages of *Penaeus duorarum*: a_1 – first antenna; a_2 – second antenna; en – endopod; ex – exopod; fr – frontal organs; fu – furca; lm – labrum; md – mandible; mx1 – first maxilla; mx2 – second maxilla; mxp1 – first maxilliped; mxp2 – second maxillipd; o – oscellus; sc – scaphognathite (from Dobkin, 1961).

a) Nauplius I: Body pear-shaped.
b) Nauplius II: 1 long, 1 moderate and 1 short terminal setae on 1st antennae.
c) Nauplius III: 2 distinct furcal processes, each with 3 spines.
d) Nauplius IV: Each furcal process with 5 spines (1); segmentation of appendages apparent (2); 1st and 2nd maxillae and maxillipeds present (3).
e) Nauplius V: Body more or less depressed; swollen knoblike structures at bases of mandibles present (1); frontal organs present (2).

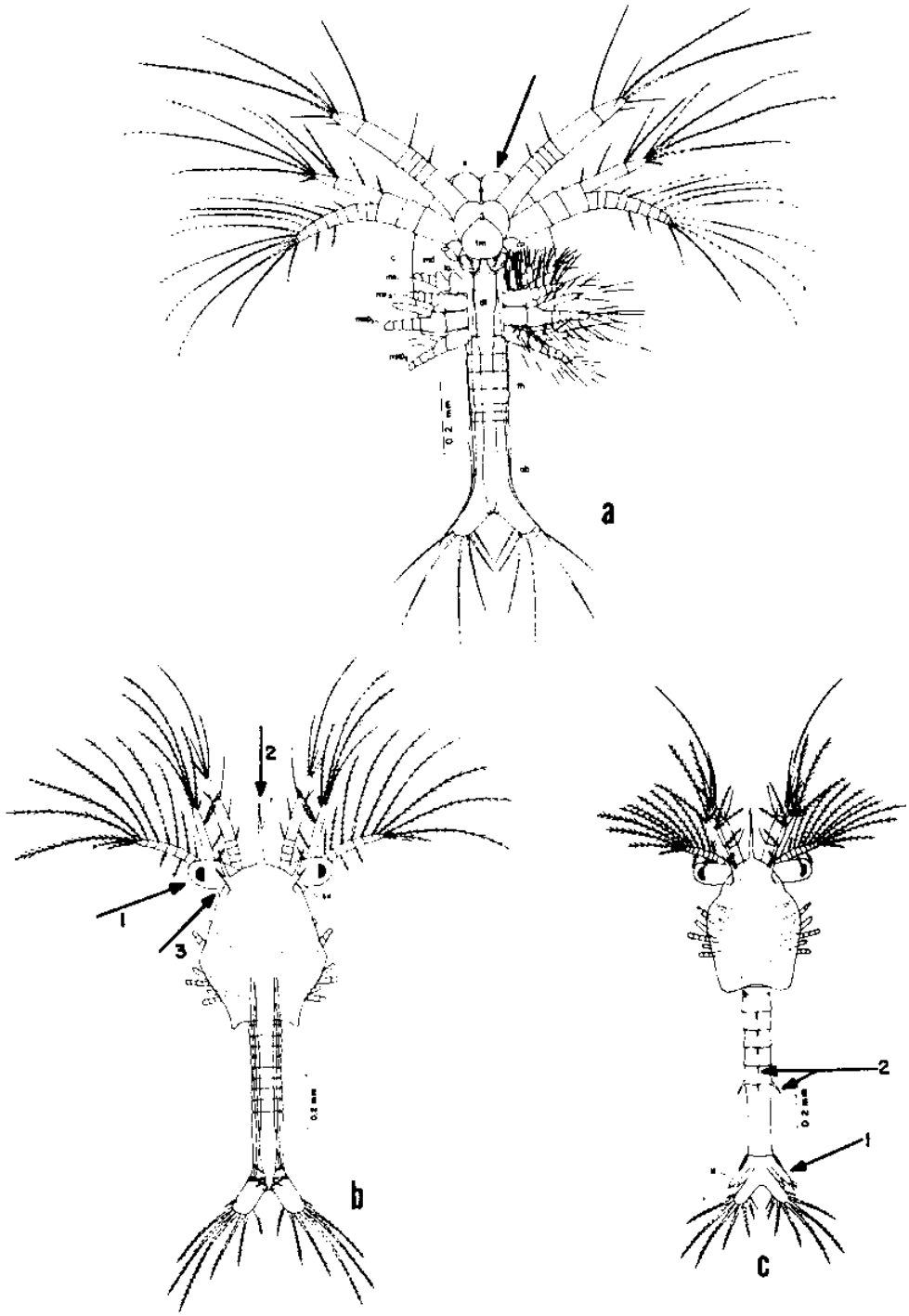


Figure 9 Zoeal substages of *Penaeus duorarum*: ab – abdomen; c – carapace; dt – digestive tract; e – eye; la – labium; md – mandible; mx1 – first maxilla; mx2 – second maxilla; mxp1 – first maxilliped; mxp2 – second maxilliped; r – rostrum; su – supraorbital spine; th – thorax; u – uropod (from Dobkin, 1961).

a) Zoea I:
b) Zoea II: Eyes stalked (1); rostrum present (2); supraorbital forked spines present (3).
c) Zoea III: Pair of biramous (doubly branched) uropods developed (1); spines appear on abdominal somite (2).

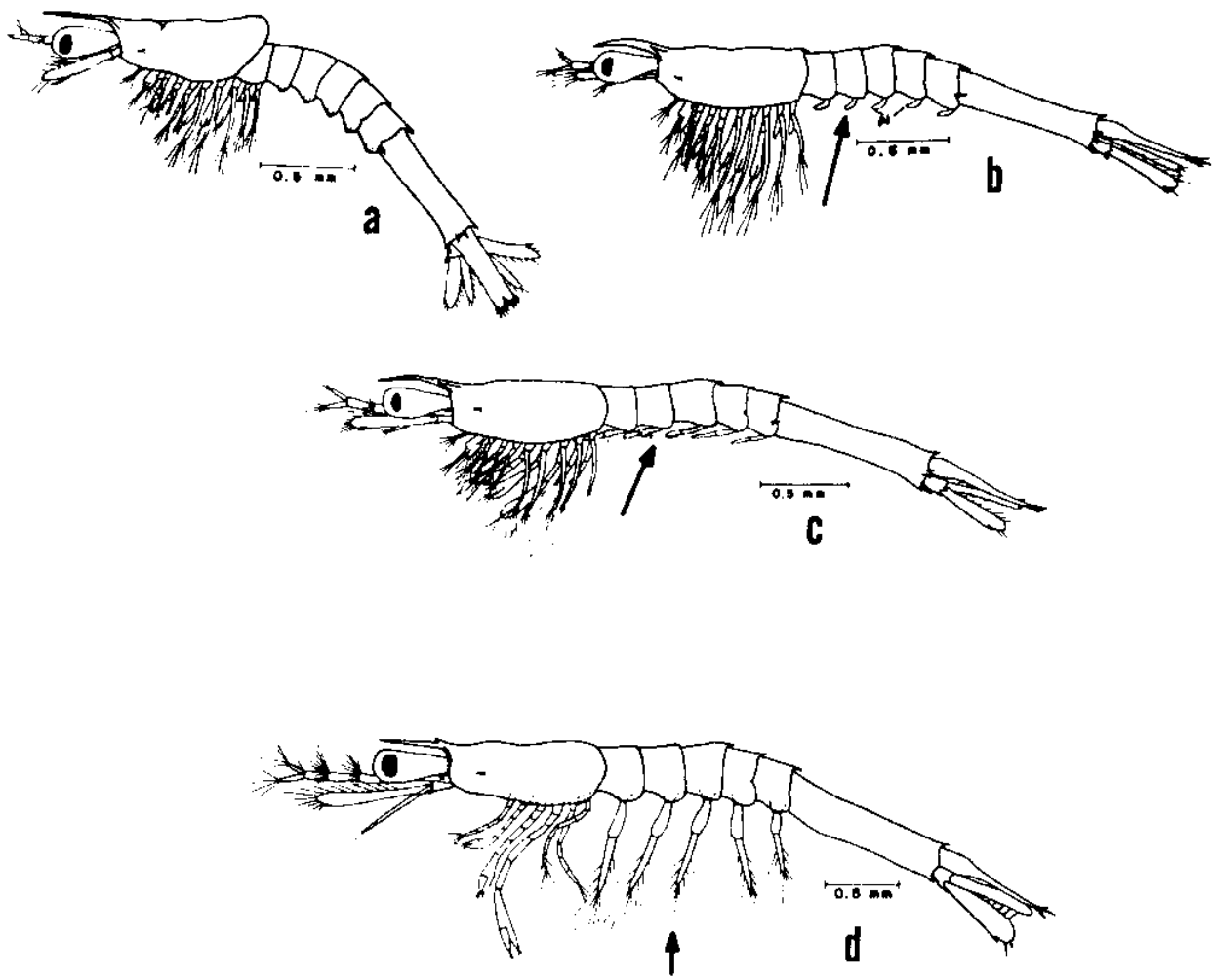


Figure 10 Myses and Postlarval substages of *Penaeus duorarum*: p1 – pleopod (from Dobkin, 1961).

a) Mysis I: Shrimp-like body structure.
b) Mysis II: Pleopod buds apparent but unsegmented.
c) Mysis III: Pleopods elongated and segmented.
d) Postlarva I (PL_1): Swimming setae present on pleopods.

EXERCISE I. DAILY LRT INSPECTION

OBJECTIVES:

1. To make an initial visual inspection of the larvae, the larval rearing tank and the supporting systems. This will include a judgment of the larval stage and health, the water quality and temperature, the light, heat and air supply and the amount of food in the tank.
2. Inspect at least three of the larvae under the dissecting or compound microscope. Accurately judge and record the substage. Inspect for and record any deformities or contamination.
3. Read and record the water temperature, salinity and pH. Take action, should these be unacceptable.
4. Perform routine water exchange.
5. Count and record larval food remaining in the LRT (algal cell density and/or *Artemia* concentration).
6. Calculate, record and administer additional feed requirements.

MATERIALS AND EQUIPMENT:

16-liter larval rearing tank, including larvae (100 per liter) and all support systems.

500 ml beaker.

Eye dropper or pipet.

Glass slide and cover plate.

Dissecting microscope.

Compound microscope.

Daily record sheets.

Thermometer.

Refractometer.

pH meter.

Siphon tube with filter (see Figure 3[b], Page 4).

Two buckets (one for discarded and the other for fresh seawater).

Hemacytometer.

METHODS:

(1) The initial inspection will not be easy for the neophyte aquaculturist. However, within a few days, a very quick inspection of all the tanks will allow him/her to recognize possible problem areas. Immediate closer inspection and action can then be taken.

Check the rate of aeration. This should be a steady flow of little bubbles, but should not "boil." Put your hand against the side of the tank to judge the temperature. It should feel a little cooler than your hand. Fold and squeeze the air supply tube, so as to cut off the air. Inspect the quality of the water. It should be clear with a slight yellow or green tinge, if algae is being fed. It should be neither milky nor dark yellow and there should be no strands of algae or bacteria developing on the sides of the tank.

Using the identification charts (Table I, Pages 13 and 14, and Figures 7 through 10, Pages 15 through 18) and with a little experience, the major stage of metamorphosis (nauplius, zoeal or mysis) can be distinguished with the naked eye. Notice the size, shape and swimming characteristics. The condition of the larvae can also be judged. Are they swimming as they should (if at all!)? Note the presence or absence of fecal threads, which will indicate whether or not they are eating well.

(2) To remove a sample from the tank, scoop some water with a clean 500-ml beaker. Use the eye dropper or pipet to draw up the larvae and place them, in single drops of water, on the glass slide. It might be interesting to look at the animals under the microscope before killing them, as they might become discolored, distorted or damaged when killed. A drop of Lugol's stain can be placed on each animal. They are extremely sensitive and die quickly.

The general condition of the animal should be inspected, with particular attention to the appendages. Setae should be straight and whole. Disfigurement might include curled, kinked setae or appendages. Bacteria (a sign of poor water quality and contamination) may also be present, and may appear in the form of debris or as described in the larval diseases section on Page 61. Side effects of bacteria on larvae may cause numerous symptoms, including localized necrosis or discoloration on any appendage, or a yellow-vermillion and red color permeating the entire nervous system. The muscles should be reasonably translucent and not milky or discolored, brown or have "little bubbles" in them (signs of disease). Is the hepatopancreas full and is the gut full and straight? At very high magnification, are there signs of bacterial colonization of the gills, setae, etc.?

Should abnormalities be found, further samples may be taken to gauge the extent of the problem. Once again, the beginner will soon learn what a healthy animal should look like and experience will lead to ease in identifying abnormalities.

The stage and substage of the larvae can now be determined (see Table I, Pages 13 and 14, and Figures 7 through 10, Pages 15 through 18). Record the above information on the "Larval Rearing Tank Data Log" for each tank (Figure 4, Page 7).

(3) Measure and record the temperature. Ideally, it should be 28°C. Should it be more than 1°C above or below this, the thermostat should be adjusted, as described in Chapter II, "Introduction to the Laboratory," on Page 5.

The salinity is read with a refractometer and recorded. This should be 32 (±4) ppt. Should it be outside the range of 28 to 36 ppt., water exchange or slow dilution with distilled water should be carried out.

A pH meter is used to tell if the medium sample is acidic, basic or neutral. The optimum pH is 8.0. If outside the 7.6 to 8.4 range, water exchange is used for correction.

NOTE: TO PREVENT DAMAGE, CARE SHOULD BE TAKEN NOT TO IMMERSE THE pH ELECTRODE DEEPER THAN INDICATED ON THE INSTRUMENT.

Should the pH or salinity levels be unacceptable, the water exchange required could exceed 50 percent. The tank should then be monitored closely for the next 24 hours.

(4) Routine water exchange is between 25 percent and 33 percent per day.

NOTE: TO PREVENT THE IMMERSION HEATER FROM BURNING OUT, IT SHOULD BE UNPLUGGED BEFORE THE TANK IS DRAINED.

Before the water is removed, the aeration can be increased so as to agitate the water and mix the heavier particles into suspension. The filter end of the siphon is then lowered into the tank and approximately 5 liters is siphoned into the "discard" bucket. Now the aeration is decreased to the normal level.

Before the water is replenished, the temperature and salinity of the fresh seawater is checked. Should it be unacceptably higher or lower than that of the tank, it must be adjusted or be added slowly so as to acclimate the larvae. The rate of change should not exceed 1°C or 1 ppt. every 15 minutes. Return the immersion heater to the tank and plug it in. Adjust the aeration.

(5) To ascertain the amount of food remaining in the LRT after the water change, refer to two other exercises. If algae is to be fed, see Exercise III, Page 45. If *Artemia* are to be fed, see Exercise IV, Methods 3, 4 and 5, Page 54.

(6) Refer to Table II, Page 22, for food requirements of each larval stage. To calculate the amount of food to be added to the LRT, refer to the same two exercises as in "5" above.

A thorough record of all larval and LRT conditions should be kept. This will not only improve the management of that particular batch of larvae, but also allow better preplanning and "fine tuning" of future work.

Table II
LARVAL REARING FEEDING REGIME
(from Treece, 1985)

Days of Larval Culture	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Average Hours Range (hrs)			48 hrs (36-51)		40 hrs (36-48)		40 hrs (36-48)		40 hrs (36-48)		24 hrs	24 hrs	24 hrs	24 hrs	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24
Larval Stage	Eggs Hatch 14 hrs @ 28°C		N_1 to N_3	N_4 to N_5	P_1		P_2		P_3		M_1	M_2	M_3	PI_1	PI_2	PI_3	PI_4	PI_5	PI_6	PI_7	PI_8	PI_9	PI_{10}	PI_{11}	PI_{12}	PI_{13}	PI_{14}	PI_{15}	PI_{16}	PI_{17}	PI_{18}
Phytoplankton (1×10^5 cells/ml) Minimum					Highest Feed Levels Maintained Cell Count Minimum 1×10^5 cells/ml																										
Artemia/ml										0.25	1	3	6	8	6	3	1	1													
Ground Pellet or Flake															Flake or ground pellet diet fed until PI's are sold or stocked (any excess *Artemia* are fed to PI's)														
Full Size Pellet																															

NOTE: Almost always 11 days from $N_3 \rightarrow PI_1$ (28°C), but the periodicity of each stage may vary considerably during this 11 day span.

IV. PHYSIOLOGY

Details of all the external parts of an adult shrimp can be seen in Figures 11, 12 and 13 on Pages 25, 26 and 27, respectively. Details of the male and female shrimp reproductive systems can be seen for open thelycum or non-grooved animals in Figures 14, 15 and 16 (Pages 28, 29 and 30, respectively). The egg development sequences can be seen in Figure 17, Page 31.

A "quick" Identification Chart of selected species may also be useful when sourcing for broodstock and can be seen on Figure 18 on Page 32.

MALE REPRODUCTIVE SYSTEM (from Motoh, 1981): The male genital system consists of internal organs; paired testes, paired vas deferens, paired terminal ampoules, and external organs; a petasma and a pair of appendix masculina.

The testis, an unpigmented and translucent organ, is composed of an anterior and five lateral lobes located in the cardiac region dorsal to the hepatopancreas under the carapace. The lobes are connected to each other at their inner ends and lead to the next organ, the vas deferens. The vas deferens arises from the posterior margins of the main axis of the testis and opens to the exterior through genital pores located medially on the coxopod of the fifth pereiopod (Figure 16-2 on Page 30). Each vas deferens consists of four distinct portions: a short, narrow, proximal medial portion having a double fixture (medial vas deferens); a relatively long narrow tube (distal vas deferens); and a muscular portion (terminal ampoule): see Figure 16-1 on Page 30.

The terminal ampoule, a bulbous structure, possesses a thick muscular wall lined with extremely tall columnar epithelial cells. It has two chambers internally; one containing the spermataphores and the other, calcareous material of a slightly gray color. The paired terminal ampoules open at the base of the coxopod of the fifth pereiopods. The spermatazoan, a minute globular body, is composed of two parts: head and tail (Figure 15-3 on Page 29). The head is large and almost circular in outline, being about 3 microns in diameter, while the tail is relatively thick and short. Although it is logical to assume that the spermatazoan is capable of movement, it has never been observed.

The petasma is a pair of endopods of the first pleopods. It is formed by the interlocking of minute hook-like structures (Figure 16-3 on Page 30).

The shape of the appendix masculina which is located on the endopod of the second pleopod is generally oval (Figure 16-4 on Page 30).

The spermatophores, one from each terminal ampoule, become fixed together longitudinally at time of extrusion and are referred to then as the "compound spermatophore".

Spermatophore structures vary considerably among closed thelycum and open-thelycum shrimp, but mating and courtship rituals are similar within each group. Spermatophores among the open thelycum shrimp are quite complicated.

FEMALE REPRODUCTIVE SYSTEM (from Motoh, 1981): The female reproductive system consists of paired ovaries, paired oviducts and a single thelycum; the first two are internal and the last is an external organ. The ovaries are partly fused, bilaterally symmetrical bodies extending in the mature female for almost its entire length, from the cardiac region of the stomach to the anterior portion of the telson (Figure 14-2 on Page 28). In the cephalothoracic region the organ bears a slender anterior lobe and five finger-like lateral projections. A pair of lobes, one from each ovary, extends over the length of the abdomen. The anterior lobes lie close to the esophagus and cardiac region of the stomach. The lateral lobes are located in the large mass of hepatopancreas and ventrally in the pericardiac chamber. The abdominal extensions lie dorso-lateral to the intestine and ventro-lateral to the dorsal abdominal artery.

The oviducts originate at the tips of the sixth lateral lobes and descend to the external genital apertures hidden in the earlike lobes of the coxopods of the third pair of pereiopods (Figure 15-2 on Page 29 and Figure 14-3 on Page 28).

The thelycum is located between a pair of the fifth pleopods and consists of an anterior and a pair of lateral plates (Figure 14-3 on Page 28).

Figure 11. Lateral View of Adult Female *Penaeus setiferus*
(From Young, 1959)

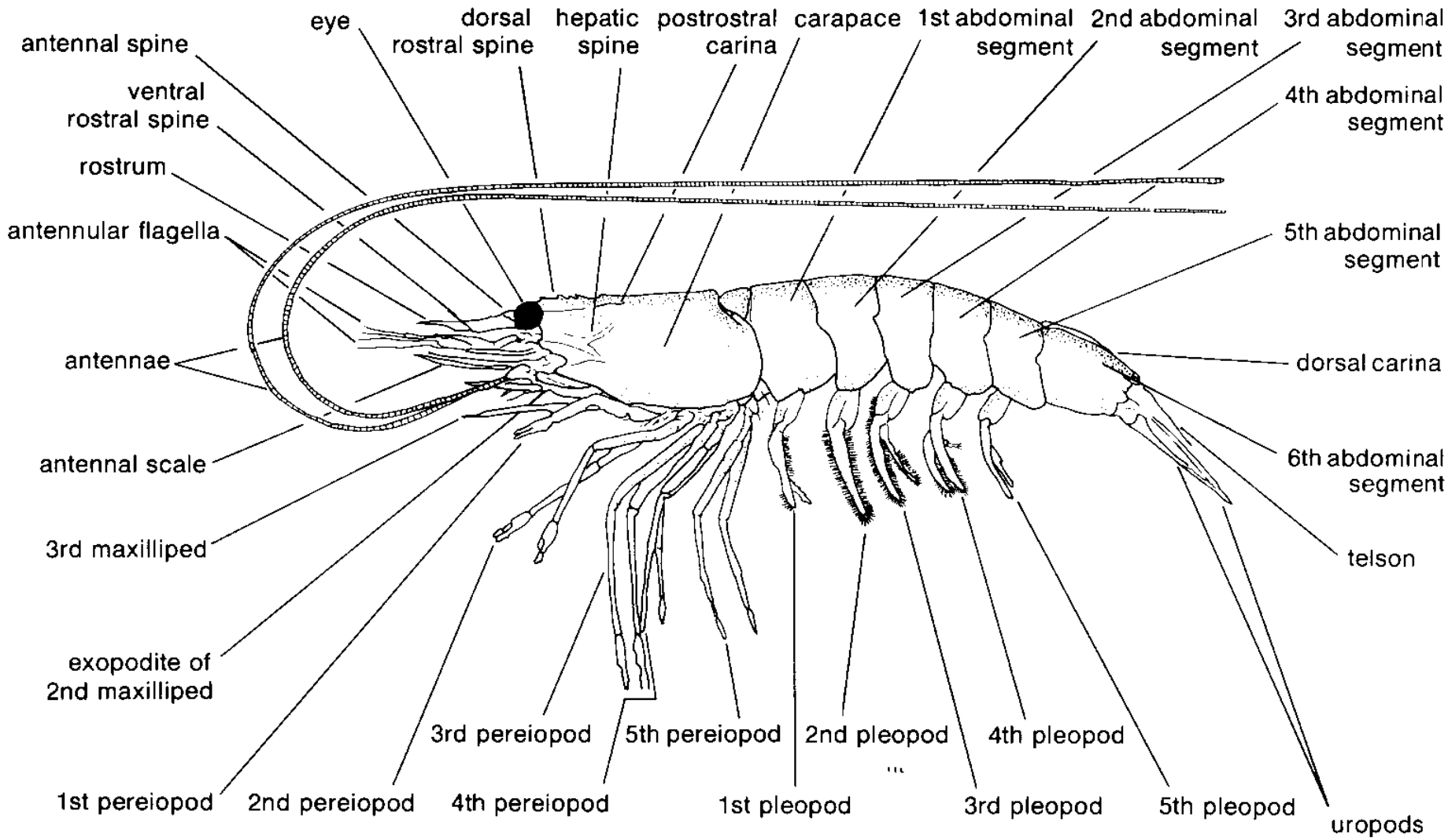


Figure 12. Dorsal View of Adult Shrimp
(From Young, 1959)

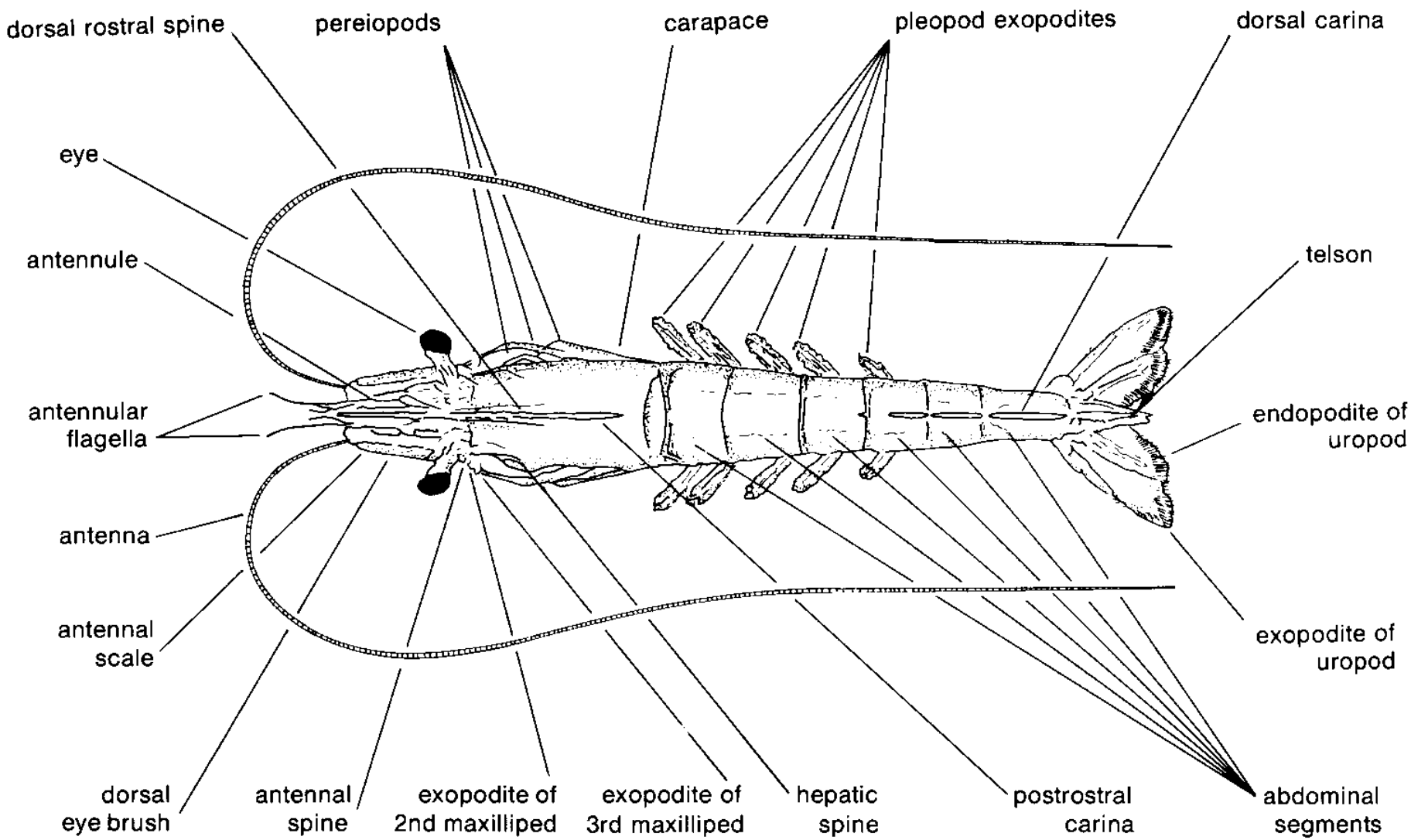


Figure 13. Ventral View of Young Male Shrimp
(From Young, 1959)

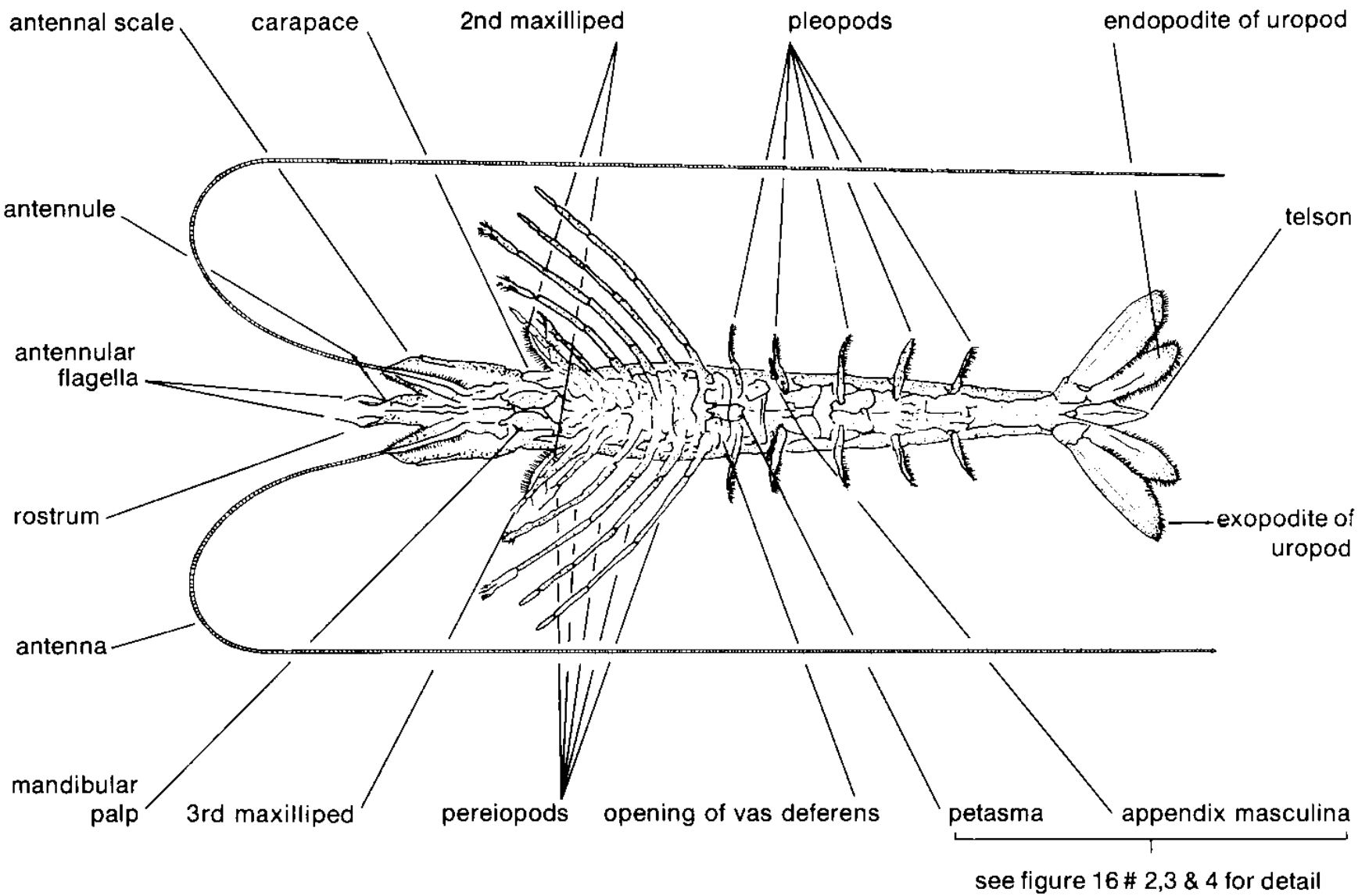
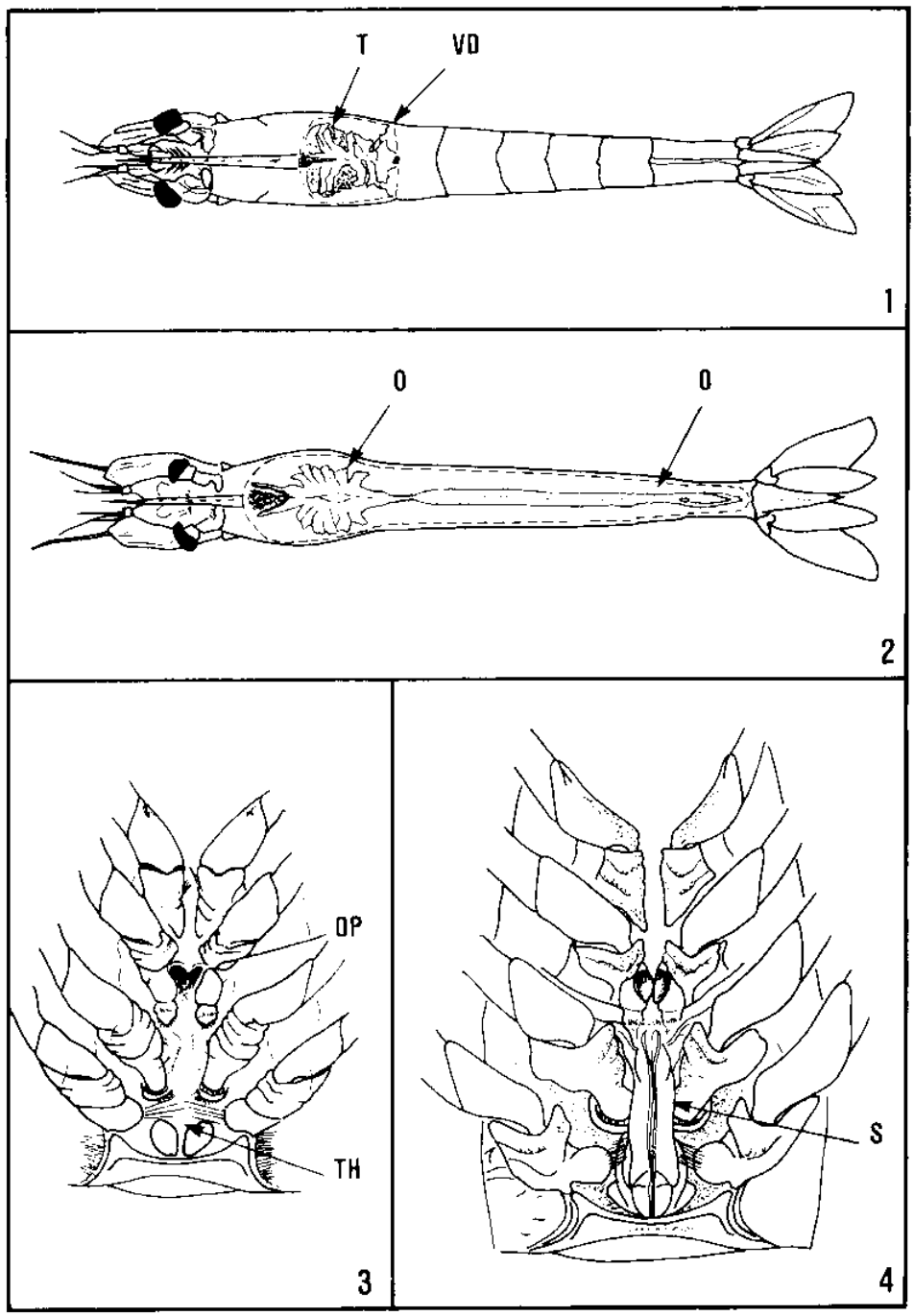
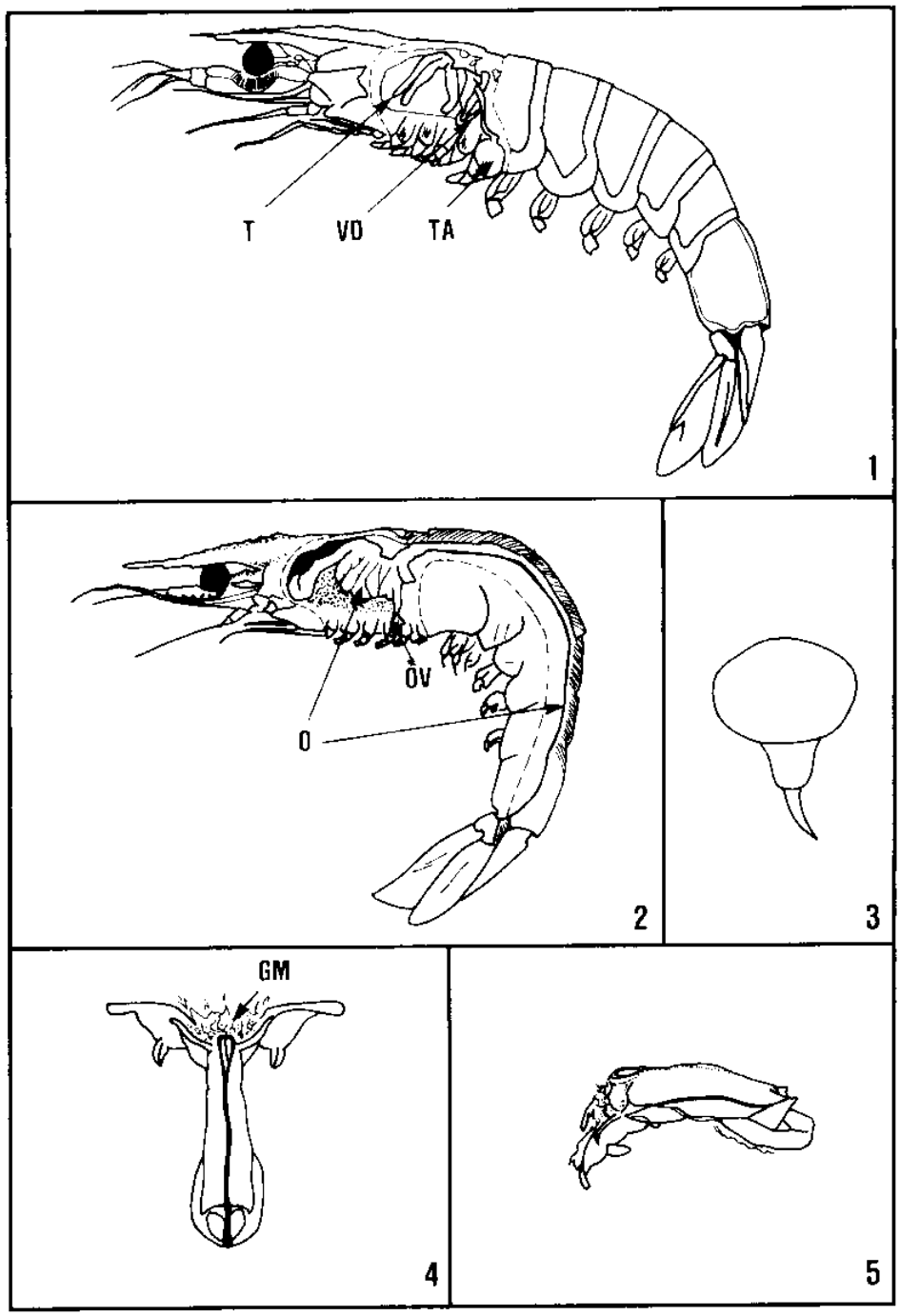


Figure 14. Details of Male and Female Reproductive System (open thelycum type or non-grooved shrimp)



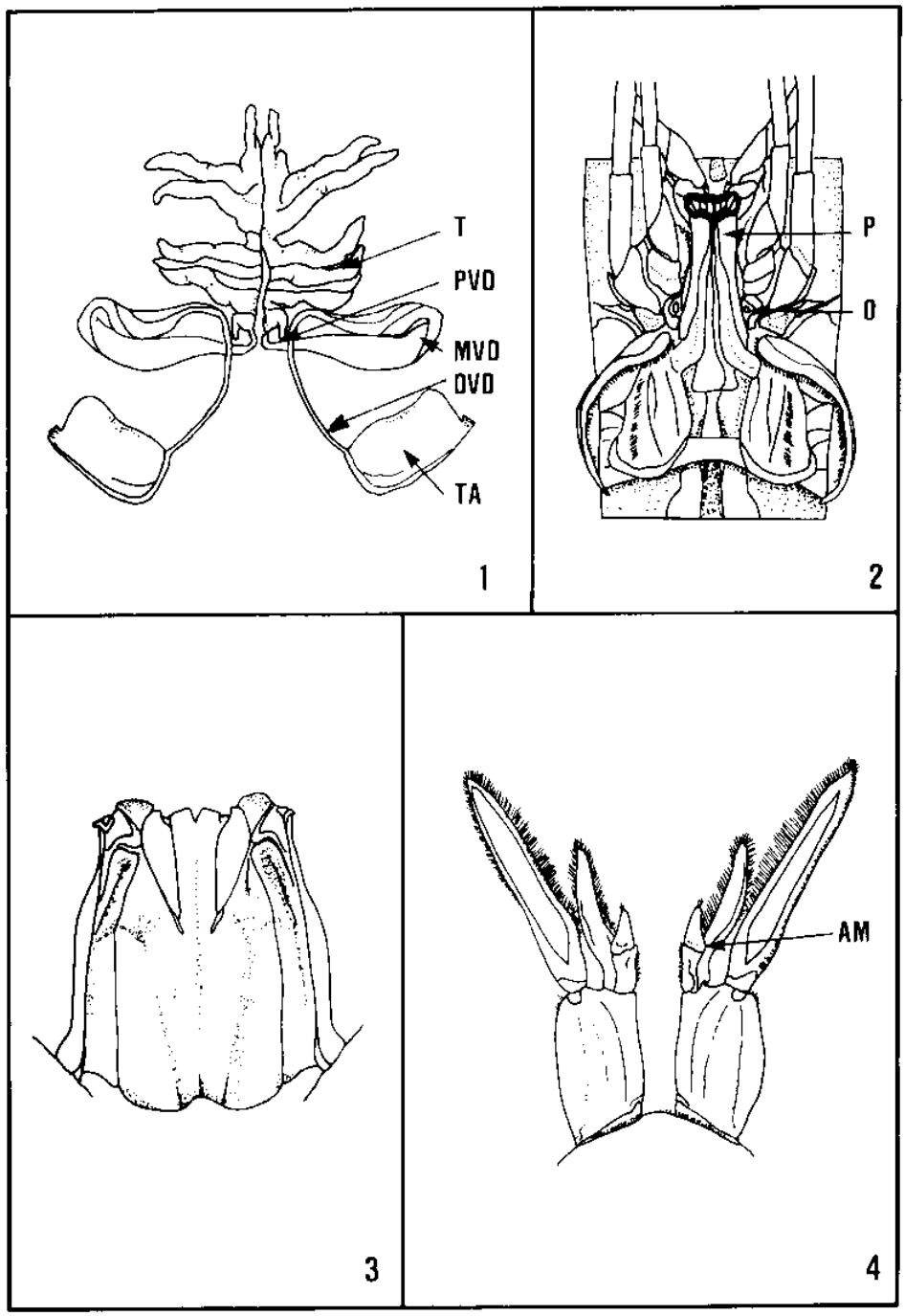
1. Diagram of male, dorsal view, dissected to show testes and portions of vasa deferentia. T-testis; VD-vas deferens. ×0.5.
2. Diagram of female, dorsal view, dissected to show ovaries. O-ovary. ×0.5.
3. Diagram of ventral surface of cephalotharax of female. OP-opening of oviduct; TH-thelycum. ×2.5.
4. Diagram of ventral surface of cephalotharax of female with spermatophore attached. S-spermatophore. ×2.5.

Figure 15: Details of Male and Female Reproductive System
(open thelycum type or non-grooved shrimp)



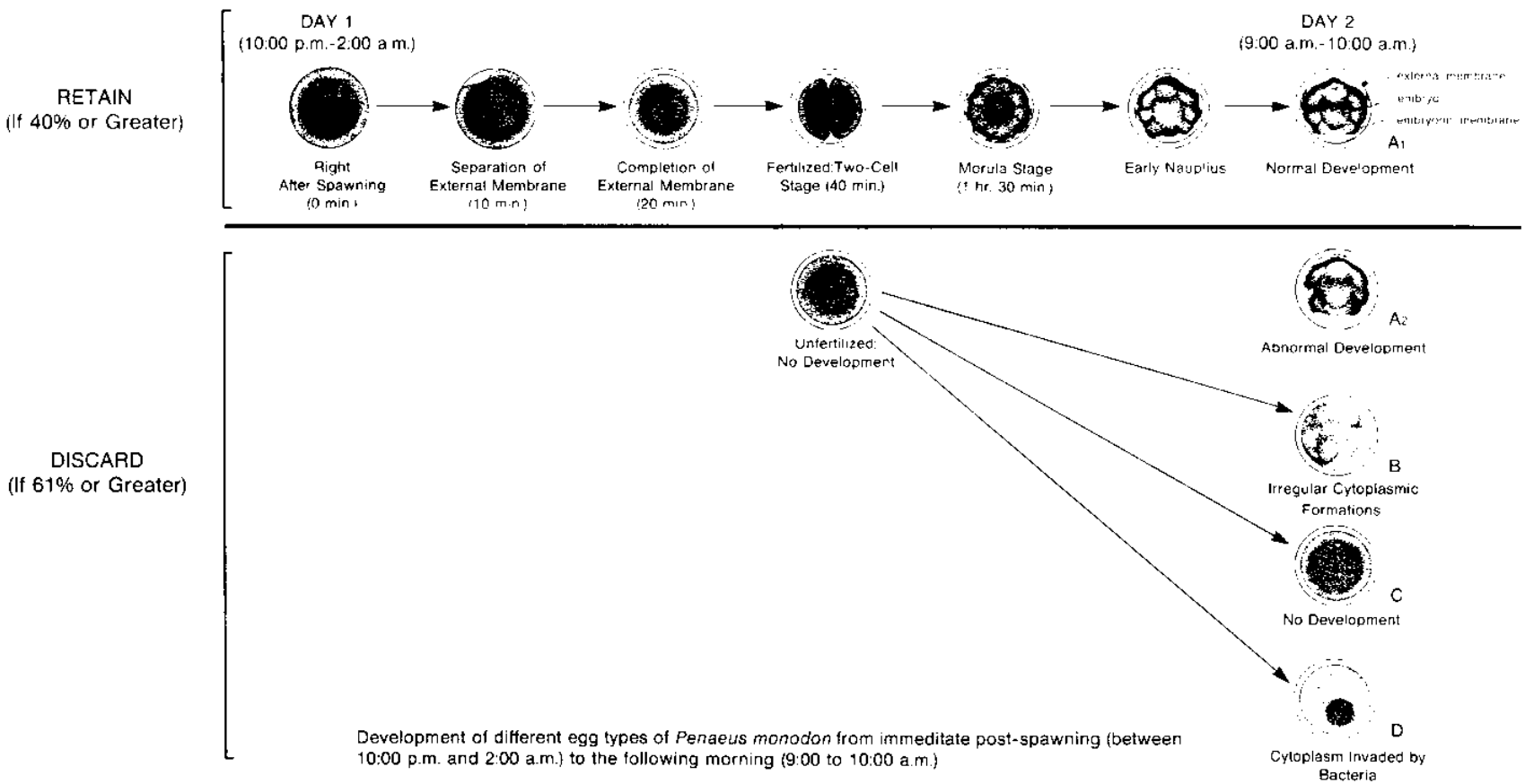
1. Diagram of male, lateral view, dissected to show reproductive organs. T-testis; VD-vas deferens; TA-terminal ampoule. ×0.5.
2. Diagram of female, lateral view, dissected to show relationship of ovary and oviduct. O-ovary; OV-oviduct; ×0.5.
3. Diagram of shrimp spermatozoan. ×9000 (approx.)
4. Diagram of ventral view of spermatophore (as in attached position). GM-gelatinous material. ×2.75.
5. Diagram of lateral view of spermatophore. ×2.75.

Figure 16: Details of Male Shrimp Reproductive System



1. Diagram of male reproductive system. T-testis; PVD-proximal vas deferens; MVD-medial vas deferens; DVD-distal vas deferens; TA-terminal ampoule. ×1.75
2. Diagram of ventral surface of mature male. P-petasma; O-opening of vas deferens. ×1.75.
3. Diagram of petasma of mature male spread open to show interior arrangement of folds. ×2.9.
4. Diagram of second pair of pleopods of male. AM-appendix masculina. ×1.75.

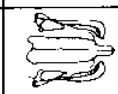
Figure 17



Development of different egg types of *Penaeus monodon* from immeditate post-spawning (between 10:00 p.m. and 2:00 a.m.) to the following morning (9:00 to 10:00 a.m.)

Figure 18

"Quick" Identification to Selected Species

SPECIES	Groove	Teeth on Rostrum (Mode)	Shape of Rostrum and Location of Teeth	1st Antennae	Thelycum ♀	Petasma ♂	Other Characteristics
P. occidentalis	no	$\frac{9-12}{3-5}$ (9/4)					Rostrum curves upward and species is easily confused with *P. stylirostris*. It has only one spot on ventral portion of tail. "Western White Shrimp"
P. stylirostris	no	$\frac{7-8}{3-6}$ (7-4-5)					Blueish pereiopods (walking legs). 2 spots on tail (one ventral, one dorsal) "Blue Shrimp"
P. vannamei	no	$\frac{8-9}{1-2}$ (8/2)					Surface of carapace is not as smooth as the other sp. when young. Red antennae. No coloration on telson, uropod region. "Whiteleg"
P. setiferus	no	$\frac{4-10}{0-3}$ (8/2)					Black coloration on telson, uropods. Rostrum long, slender, sometimes turning up. Ventral teeth apart. "Northern White"
P. schmitti	no	$\frac{7-10}{1-3}$ (8/2)					Petasma without diagonal crestion inner surface of distal part of lateral lobe. Thelycum has parallel crests. "Southern White"
P. duorarum	yes	$\frac{7-10}{1-3}$ (8/2)					Dorsolateral grooves of sixth abdominal somite narrow "Northern Pink"
P. aztecus	yes	$\frac{5-10}{0-3}$ (8/2)					Median groove of postrostral carina long and deep. Dorsolateral grooves broad, "Northern Brown"
P. brasiliensis	yes	$\frac{7-11}{0-3}$ (8/2)					Petasma with long projections and distal folds which penetrate deeply into petasma. "Red Spotted"
P. californiensis	yes	$\frac{8-9}{2}$					Saddle-like structure on posterior portion of 3rd abdominal segment and anterior part of 4th segment rostrum straight. Exterior coffee colored (dorsal posterior and ventral) "Yellowleg"
P. brevirostris	yes	$\frac{8-10}{2-3}$					Last ventral tooth anterior to or on same level as first dorsal tooth. No longitudinal carina on thelycum. Dark pink. "Crystal Shrimp"
P. monodon	yes	$\frac{7}{2-3}$ (7/2)					Groove (rostral) is shorter than in *P. semisulcatus*. "Giant Tiger"
P. semisulcatus	yes	$\frac{7}{2-3}$					Multi-colored shrimp (blue, red, yellow on swimmerets. Banded antenna (dark, light). "Green Tiger"
P. indicus	yes	$\frac{5-7}{0-1}$					Gastrorbital carina posterior 2/3 hepatic spine and orbital angle. Rostral crest high. "Indian White"

V . EYESTALK ABLATION

In the female shrimp, the production and storage sites of the gonad-inhibiting hormone are found in the eyestalk. This hormone inhibits the maturation of the ovaries. In nature, some environmental factor or factors cause the decrease of this substance as the shrimp migrate from the estuaries to offshore areas, where they normally spawn. Eyestalk ablation or extirpation eliminates or at least reduces this inhibitory hormone to a level where full maturation of the ovaries can take place.

Shrimp should be ablated only when hard-shelled, never when in post-molt (newly-molted or soft-shelled) or premolt (ready-to-molt, with white spots on shell) stages. The procedure is as follows:

(1) Hold the shrimp gently but firmly with one hand, preferably in the water. Only females are ablated.

(2) Ablation is performed on either left or right eye. However, an already infected or otherwise damaged eye should be ablated to leave one unablated healthy eye.

(3) Ablation may be performed in any of the following ways:

   (a) Pinching – Grasp the eyestalk, just behind the eyeball, between the thumb and index finger. Squeeze hard and roll the the thumb and finger outwards, thus crushing the eyestalk and squeezing out the contents of both it and the eye. The objective is to squeeze the contents outwards and not let them follow the eyestalk back into the head region. An incision on the front of the eye with a sharp blade may be made to aid this process. Some people prefer this method; however, it is merely a matter of preference. We have found the incision to be unnecessary, but prefer to hold the shrimp underwater and get the process over with as fast as possible, thus minimizing stress.

   (b) Ligation – A piece of string is tied around the base of the eyestalk, close to the carapace. The eye should fall off in a few days (Primavera, 1985).

   (c) Cautery – The eyestalk is ablated either by electrocautery or with a silver nitrate bar.

(4) To minimize stress, the ablation should be performed as quickly as possible and can be done under chilled water. However, in commercial hatcheries ablation is usually done in the early mornings, when temperatures are lowest. Female mortality due to ablation should be very low, but some mortality should be expected.

Pinching is the preferred method of ablation. It can be done by one person and the wound will heal rapidly, without requiring antibiotics. Ligation requires two persons, one to hold

the shrimp and the other to tie the eyestalk. Cautery requires either a cauterizer or silver nitrate bar, neither being easily available. Egg development in some females should become visible three days after ablation and the first spawn should occur approximately one week after ablation. The tank should be in full production three weeks after ablation. If ablated during the inter-molt stage, the females will mature and spawn immediately. However, if ablated during early premolt, they will molt first, before maturing.

Some researchers have been able to obtain egg development, mating and spawning without ablation, utilizing temperature and photoperiod manipulation, but no one has yet been able to base a long-lasting, profitable, highly productive commercial operation on this approach. Researchers are now attempting to characterize and isolate the hormones involved in maturation and offer future hope in eliminating this presently needed step. To eliminate the ablation process may also eliminate some of its side effects (fewer fertile eggs and larvae per spawn with ablation and brood animals "burning out" after three months). But until such time as ablation can be successfully eliminated and replaced with a superior method, hatcheries must depend upon it to sustain production.

VI . ALGAE

Introduction

The algae comprise a large heterogeneous (differing in structure, form, etc.) assemblage of plants. The characteristics by which they can be defined are difficult to specify. In many of the so-called simpler types of algae, organization of the cell is much more complex than that of any cell of the so-called higher plants. Reproductive processes may involve vegetative, asexual and/or sexual mechanisms. Many possess the animal attribute of motility and in form grade into the Protozoa (unicellular animals) until no distinction between the two groups is possible.

The algae have a long fossil history, some of them possibly extending back to the time of the origin of photosynthetic cellular plants. They are generally considered to be the group from which more complex plants arose. Morphologically (in form and structure), they are cellular plants growing as single cells or aggregations of cells, although these are still relatively undifferentiated into organs and only in the most complex genera are elementary conducting tissues found. Nevertheless, the range of form is great, from minute cells a few micrometers in diameter up to the large seaweeds of the Antarctic, which are many meters in length and weigh as much as a small tree.

Although generally restricted to an optimum type of habitat, some species are highly adaptive to environmental extremes. Some occur in nearly boiling hot springs (85°C). Others are to be found in snow fields. While most occur in the upper levels of fresh and marine water, some red species have been found as deep as 600 feet, at the equator.

Algae are natural food for shrimp during all but their egg and early naupliar stages. During the shrimp growout phase, less control is maintained on the algal growth and species selection is usually left to chance. However, during the larval phase, aquaculturists select species for cultivation based on the criteria of ease and cost of culture (preferably one native to the area) and on the dietary value to the larvae to be raised.

SPECIES OF ALGAE

Three species of algae that are commonly used in the culture of penaeid shrimp and other animals are: *Chaetoceros gracilis, Isochrysis* sp. (Tahitian) and *Tetraselmis chuii*.

***Chaetoceros gracilis*:** There are many species of *Chaetoceros* available as food for penaeid larvae. *C. gracilis* is a solitary marine centric diatom. It has a silicified skeleton composed of two valves (shells) which separate to form two new cells during vegetative division. It is rectangular in shape , measuring 4 to 6 micrometers exclusive of the setae. (Figure 19[c], Page 38). The cells are golden brown under the microscope. When cultured, the concentration

of cells determines the color, which ranges from yellowish-gold, at low density, to coffee-brown at high densities, above 3 x 10^6 per milliliter.

Most species of *Chaetoceros* are characterized by tolerance of high water temperature. The maximum growth temperature for *C. gracilis* is 37°C, with optimum growth at temperatures ranging between 25°C and 30°C. The mass culture of this species is generally performed within this range. The minimal salinity for its growth is 6 ppt., but it can grow well at salinities up to 50 ppt. with optimum growth occurring between 17 and 25 ppt. The growth rate of *Chaetoceros* is best under illuminations of 500 to 10,000 lux.

Tetraselmis chuii: While considered by some to be less nutritious (Enright, 1984) and a little too large for the very early zoeal stages to ingest, *Tetraselmis chuii* is easily cultured and a good food source from the second zoeal stage on. It is a green oval-shaped flagellate (capable of self-locomotion by means of flagella) ranging from 10 to 15 micrometers in diameter (see Figure 19[a], Page 38). When cultured, as the concentration of the cells increase, the green color darkens until a maximum density of approximately 500 x 10^3 cells per milliliter is reached.

Outdoor mass cultures of *Tetraselmis* grow at temperatures between 15°C and 33°C under natural light conditions. The suitable culture salinity in natural seawater ranges between 15 and 36 ppt. and for artificial seawater between 22 and 36 ppt. Although motile, *T. chuii* has a tendency to settle-out if not aerated. Here, as when the culture becomes contaminated or "crashes", the cells settle in a dark layer on the bottom and lower sides of the tank.

***Isochrysis* sp. (Tahitian):** This is perhaps one of the easiest species to culture. It is a spherical to pear-shaped, brown, naked (thin cellular walled) flagellate. It is 3 to 5 micrometers in diameter. With increasing density, the culture will change from yellowish-gold to coffee-brown at a maximum density of 3 to 7 x 10^6 cells per milliliter.

The Tahitian strain of *Isochrysis* prefers high temperatures (up to 30°C) and strong light. This species is not only tolerant of environmental changes, but appears to selectively suppress growth of undesirable contaminants (such as bacteria). While it is motile and thus able to disperse well throughout the water column, in the larger culture containers, it is generally aerated.

GROWTH CHARACTERISTICS: Before discussing the specific techniques used for growing algae, we need to understand its growth dynamics. From Figure 20, Page 39, we see that typical phytoplankton culture growth goes through five phases. When introduced to fresh medium, cells which have been taken from a sample which is in any but the exponential growth phase do not begin to grow immediately. Perhaps they require time to recover before reproducing. This is called the lag phase.

Usually, after several days they move into the desired exponential growth phase. In this phase, growth is rapid, the cells are more nutritious to the larvae and the culture seems better able to resist contamination.

With no intervention, the culture would multiply until limited by light and/or nutrition and the effects of toxins slow and then stop its growth. Finally, these limitations would cause the culture to die off or "crash". As the algae deteriorates, it becomes more and more susceptible to contamination. Further, it has lower nutritional value and its metabolites might even prove toxic to shrimp larvae.

PROCEDURES FOR ALGAE CULTURE: Maintain an environment which favors the growth of the algae and discourages that of its competitors. To keep the algae in the growth phase, use a "batch system." Transfer the culture into progressively larger containers, thus giving them more nutrients and fresh medium in which to grow. Transfer from the initial test tubes into flasks and then into 19-liter carboys (see Figures 21, 22 and 23 on Pages 40, 41 and 42, respectively).

Use seawater of approximately 32 ppt. salinity. To remove any large particles or contaminants, pass the water through filters down to 1 (or even one-half) micron. Thereafter, the water can be directed past an ultraviolet light so as to kill most bacteria and other potential contaminants.

The nutrients are now mixed into the water. The most commonly used nutrient mixes are Erdschreiber's or Guillard's F/2, but several other mixes can be used (see **Journal of Phycology**, Vol. 14 Supplement, 1978, The Culture Collection at the University of Texas, available from the Department of Botany, U.T., Austin, Texas 78712). A premixed medium from Fritz Chemical Co., Dallas, Texas, can also be used. Instructions can be found on the label. Because silicate is required by *C. gracilis*, for the production of valves and because it has no detrimental effect on the other two species, it may be simpler to add it to all the medium.

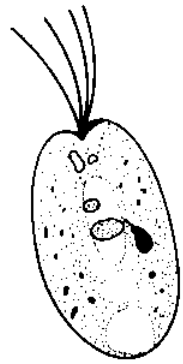
Before adding the medium to the culture containers, it is very important to thoroughly wash and rinse the containers. Some recommend that they first be soaked in H_2SO_4.

Guillard's F/2 algae culture medium consists of major nutrients (sodium nitrate, sodium phosphate and sodium silicate), trace metals and vitamins mixed in water. Composition per liter seawater is as follows:

Major nutrients:	$NaNO_3$ = 75 mg	$NaH_2PO_4 \cdot H_20$ = 5 mg	$Na_2SiO_3 \cdot 9H_20$ = 30 mg (Silicate may be omitted for species other than diatoms.)
Trace metals:	$Na_2 \cdot EDTA$ = 4.36 mg $ZnSO_4 \cdot 7H_20$ = 0.022 mg $Na_2MoO_4 \cdot 2H_20$ = 0.0006 mg	$FeCl_3 \cdot 6H_20$ = 3.15 mg $CoCl_2 \cdot 6H_20$ = 0.01 mg	$CuSO_4 \cdot 5H_20$ = 0.01 mg $MnCl_2 \cdot 4H_20$ = 0.18 mg
Vitamins:	Thiamin • HCL = 0.1 mg	Biotin = 0.5 mg; B_{12} = 0.5 mg	

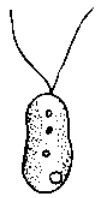
(The richer Guillard's F/1 medium has twice the amount of each nutrient per liter of seawater.)

a.



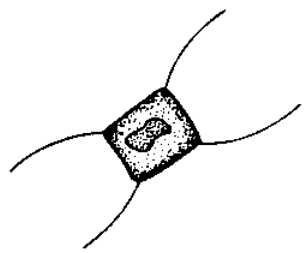
Tetraselmis sp. (10-15μm)

b.



Isochrysis sp. (3-5μm)

c.



Chaetoceros sp. (4-6μm)

Figure 19. Some Commonly Cultured Microalgae

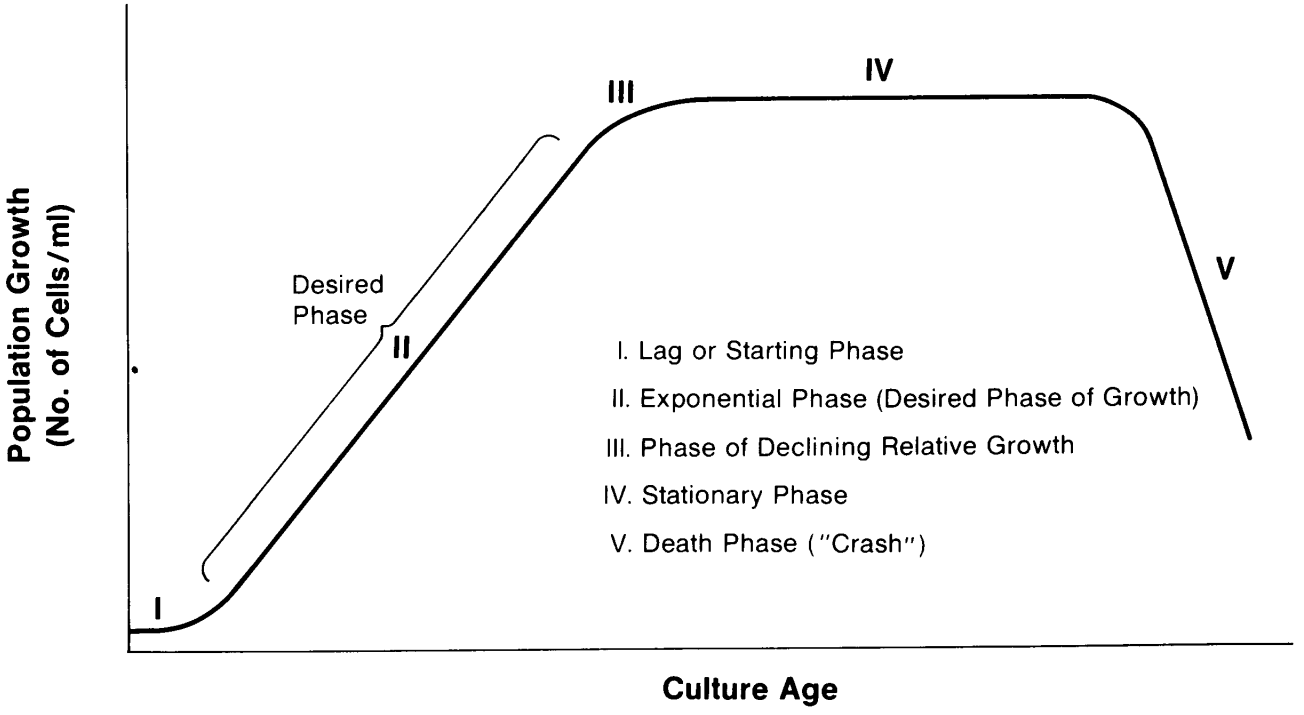


Figure 20. Typical Phytoplankton Culture Growth

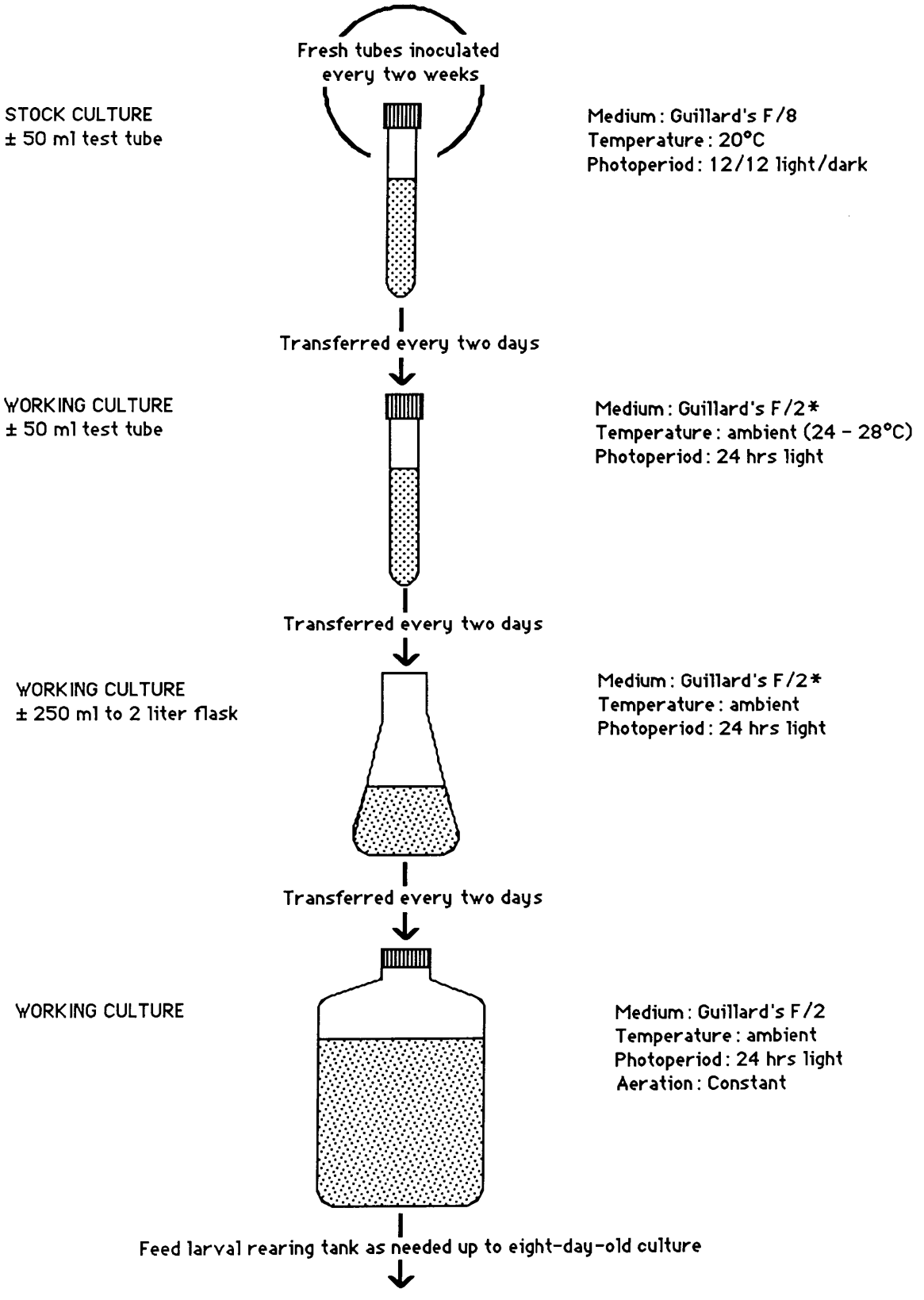


FIGURE 21: TYPICAL CYCLE USED IN ALGAE CULTURE

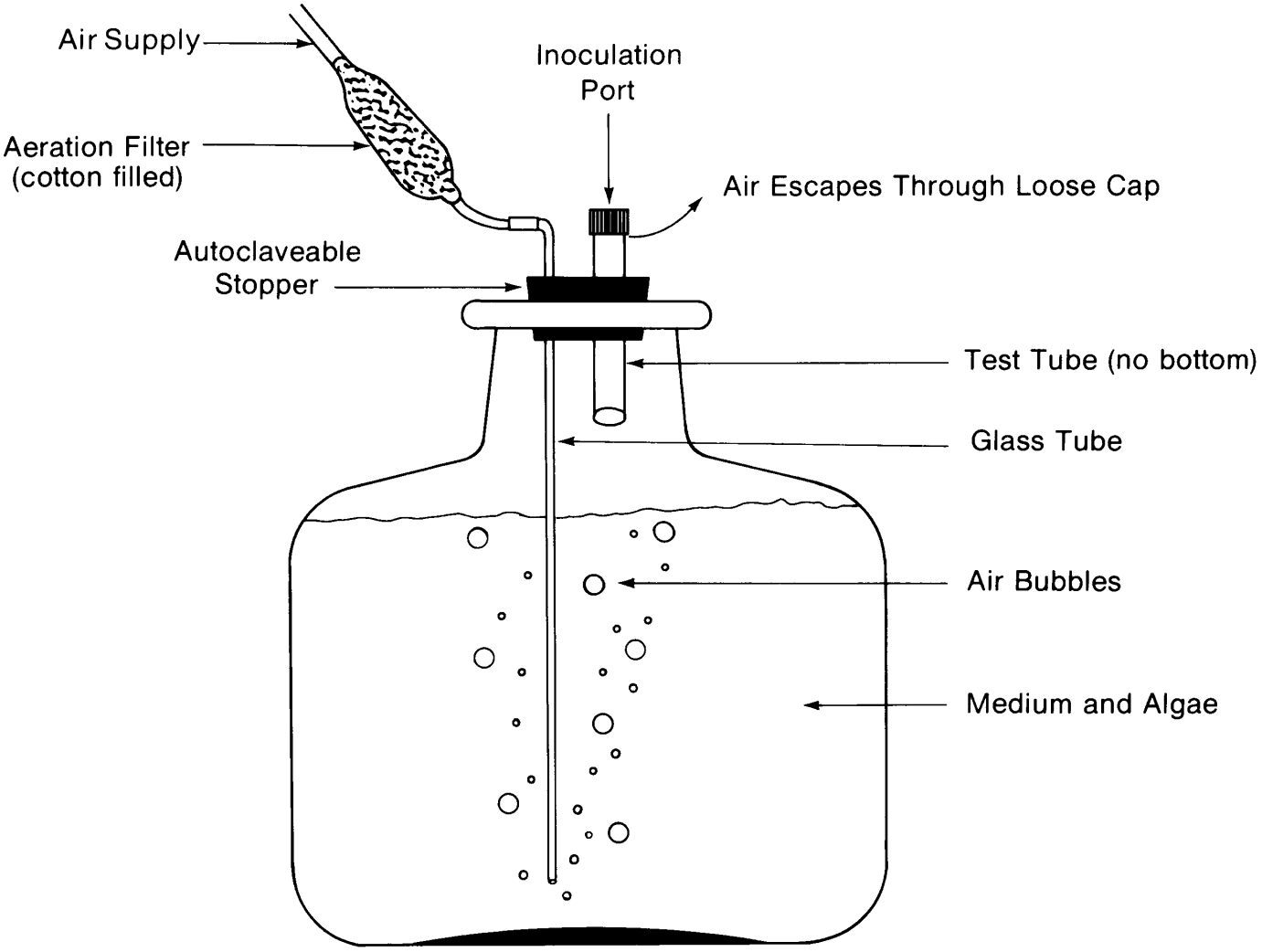


Figure 22. Glass Carboy

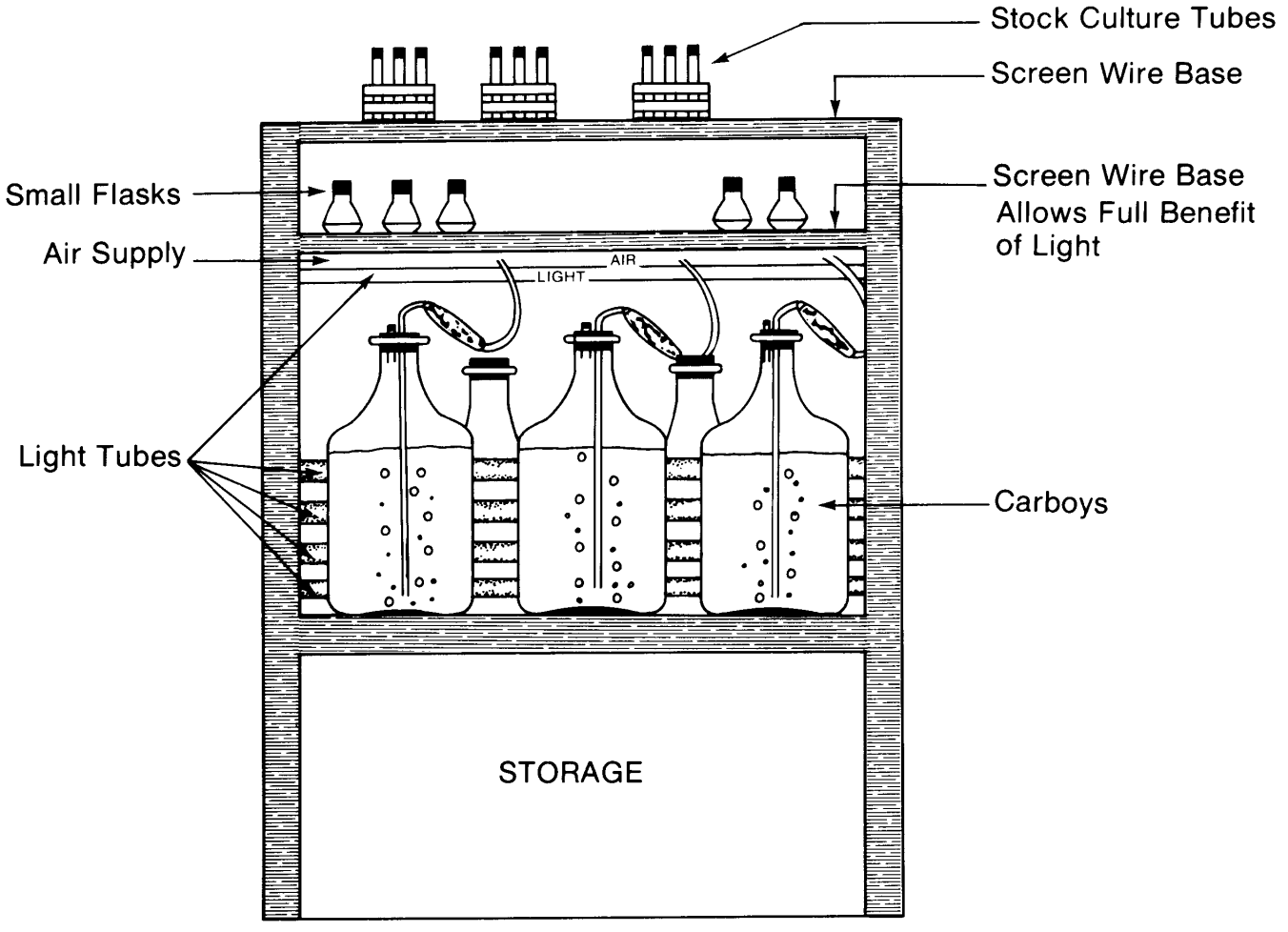


Figure 23. Typical Algae Culture Rack

EXERCISE II. IDENTIFICATION, TRANSFER AND CULTURE OF ALGAE

INTRODUCTION: Participants will be able to pick out individuals of each of three species under a microscope. They will be able to mix and sterilize a growth medium, and they will be able to aseptically inoculate this medium with algae for batch culture.

OBJECTIVES:

1. To identify and observe algae under the microscope.
2. To prepare and sterilize an algal growth medium.
3. To learn aseptic transfer and inoculation techniques.
4. To inoculate algal medium from stock cultures.

MATERIALS AND EQUIPMENT:

Sample algae cultures.
Test tube rack.
Pipet or eyedropper.
Hotplate and Bunsen burner with stand.
Glass slides and coverplates.
Compound microscope.
Sterile hood (optional).
Cleaning materials, including distilled water.
1 liter filtered seawater.
F/2 medium reagents, ready for adding to seawater.
Graduated pipet, 1-2 ml, with bulb.
2-liter flasks.
Thermometer, to 80°C-100°C.
Scissors.
Aluminum foil.

METHODS:

(1) (a) Ensure that all the above apparatus and all surfaces are clean and dry.

(b) Light burner and adjust the flame.

(c) Swirl test tube so as to agitate sample. Be sure not to allow the culture to come into contact with the cap/stopper. Ensure that the cap/stopper is loose and return it to the rack.

(d) Remove a sterile pipet/dropper from its cannister, using a pair of forceps, and hold it between the thumb and index finger with your index finger covering the top.

(e) Holding the tube in your left hand, grasp the cap/stopper with the little finger of your right hand and twist it off (finger curled around the cap/stopper).

(f) Flame the mouth of the tube with a brief rolling motion.

(g) Still holding the cap/stopper in your right hand (little finger curled around it), insert the pipet/dropper into the tube (holding it with your thumb and two fingers of the right hand) and draw off a small quantity of the sample. Try not to let the pipet/dropper touch the side of the test tube.

(h) Flame the mouth of the tube once more, twist the cap/stopper back on loosely and return it to the rack.

(2) The sample of algae can now be transferred to fresh medium for further culture, or a glass slide for inspection. First inspect the cells under a microscope.

(a) Deposit one or two drops of the medium on a clean glass slide.

(b) Inspect the cells under the compound microscope. (Those who are not familiar with the use of the compound microscope should refer to Technical Sheet IV, Page 77, before continuing with the exercise). Identify the species (if any), noting the size, shape, color and activity of the cells (see a detailed description of three species on Pages 35 and 36). Inspect for possible deterioration and contamination. This will be in the form of debris or foreign cells.

(3) (a) For medium preparation, pour one liter of filtered seawater into a 2-liter flask.

(b) Add 1 ml each of the F/2 reagents, cleaning the pipet with distilled water after each reagent.

(c) Place a square of aluminum foil on the flask and place it on the hotplate or over a Bunsen burner. Turn up the heat on the flask.

(d) When bubbles in the flask begin to move, take the temperature, and continue taking it every 2–3 minutes.

(e) When the temperature reaches 73°C, turn down the heat to keep the temperatures at 73°C for 10–15 minutes.

(f) Remove the flask and let the medium cool overnight.

(g) Repeat steps (c) – (f) 24 hours later to completely pasteurize the medium.

(4) (a) For medium inoculation, use medium that has been sterilized in advance and is at room temperature.

(b) Fill a sterile 500-ml flask approximately one-half full with medium.

(c) Add 50 mls of algal inoculum. (Alternative: add 250 ml algae to 2 liters medium.)

(d) Place inoculated medium near lights and add aeration.

EXERCISE III. COUNTING, MAINTAINING AND FEEDING ALGAE

INTRODUCTION: Participants will be able to use a hemacytometer to count algae in culture vessels and larval rearing tanks. They will be able to calculate the volume of algae necessary for each feeding and also will feed algae to the larvae at the correct amounts, and attempt to maintain these feed levels in the rearing tanks.

OBJECTIVE:

1. To count the number of algal cells per ml in culture vessels and larvae tanks.
2. To calculate the correct volume to feed larval tanks.
3. To feed larval tanks and maintain correct algal counts in tanks.
4. To maintain algal cultures and transfer to larger vessels when necessary.

MATERIALS AND EQUIPMENT:

Bunsen burner or alcohol burner.
Hemacytometer and cover glass.
Pipets.
Compound microscope.
Cleaning materials.
Prepared algal medium.
Graduated pipet with bulb.
Graduated cylinder.
19-liter culture vessels.
Lugol's iodine solution.

METHODS:

(1) (a) For counting non-motile algae, place cover glass on hemacytometer and use clean pipet to release liquid to be counted at edge of cover glass (the v-groove). Liquid should be pulled under cover glass but should not overflow or move into moats on the hemacytometer.

(b) For motile algae, place 5 ml of liquid to be counted in test tube and add a few drops of Lugol's solution or suitable substitute. Agitate tube and follow above process (a).

(c) Place hemacytometer under microscope with 10x or similar low power objective in place.

(d) Follow steps 6 and 7 of Technical Sheet I, Page 66.

(2) (a) After counts have been obtained in the algae culture vessels and the LRT (if appropriate), calculate the number of cells or volume needed to feed the larvae (rates given in Table IV, Page 65).

(b) Feeding calculations (from Wilkenfield et al., Feb. 1986, unpublished manuscript).

Desired cell density – Residual cell density *X* LRT volume
= Total number of cells to be added to LRT

$$\frac{\text{Total number of cells to be added}}{\text{Cell density in algae mass culture tank (cells/ml)}} = \text{Total volume (ml) of algae from mass culture tank that should be added to LRT to achieve desired feeding density}$$

These calculations need to be done individually for each species of algae being fed, and for each larval rearing tank. Record algae mass culture tank number, cell density and volume added to LRT on LRT Log Sheet.

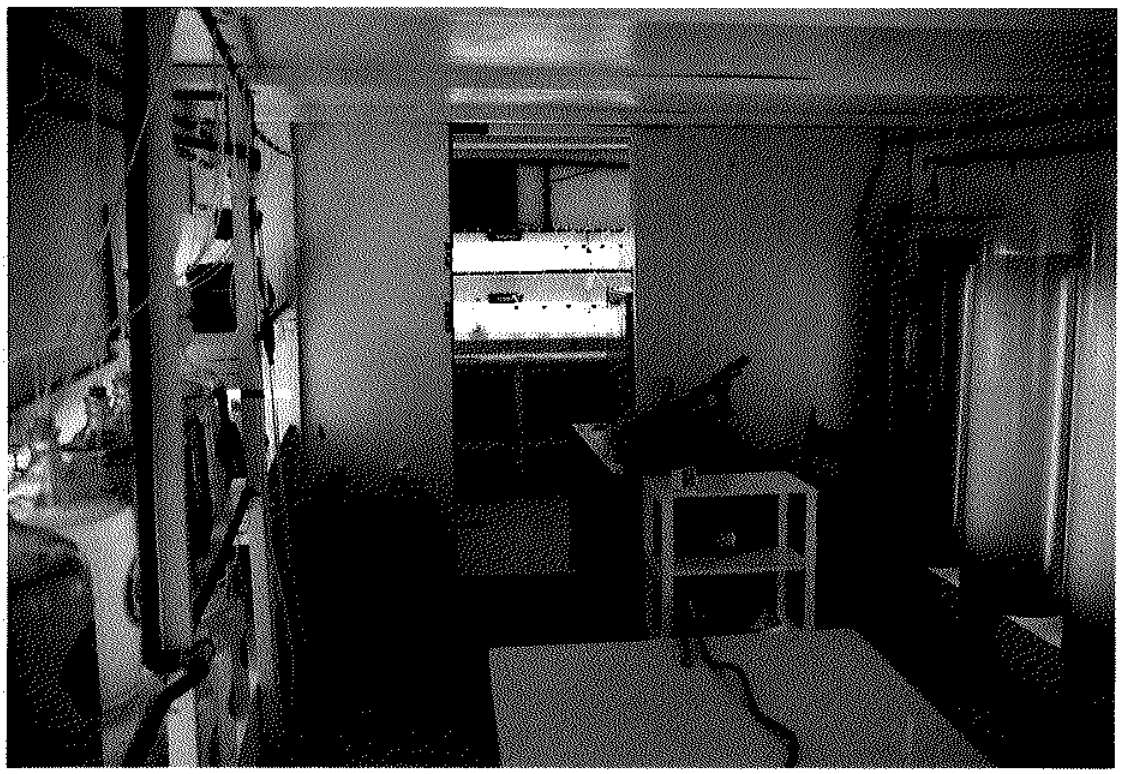
(c) Feed larval tanks, drawing off the amount of water to be added before feeding.

(3) (a) Maintain and transfer algae cultures as needed (also described earlier).

(b) Algae feeding concentrations are dependent on four factors: desired feeding density, residual (uneaten) cell density, volume of the LRT, and the cell density in the mass culture tank out of which algae is to be fed. The formulae to be used are:

A typical algae room arrangement can be seen in Figure 24, Page 47. Note the lower cell counts in the lighter-colored medium containers (yellow-to-gold) and the higher densities in the medium containers that are dark brown or, in the case of *Tetraselmis*, dark green. Also note how clean and uncluttered the room appears.

Typical Algae Room Arrangement



VII. ARTEMIA

Introduction

(Treece and Wohlschlag, 1987)

Adult *Artemia* (brine shrimp) were first described by Schlosser in the mid-1700s. Seal (1933) and Rollefsen (1939) reported the value of freshly hatched *Artemia* nauplii as food for fish fry. Thereafter exploitation of *Artemia* has gradually increased.

The value of *Artemia* in aquaculture is due to the unique characteristics of its reproduction, development and physiology (Sorgeloos and Personne, 1975). *Artemia* have two modes of reproduction: 1) ovoviviparous, when nauplii hatch in the ovisac of the mother and are born live, and 2) oviparous, when embryos at the gastrula stage of development are encased in a hard capsule, or cyst.

The dehydrated cysts can be stored for months or years without loss of hatchability (Dutrieu, 1960). The cyst is 200 to 300 micrometers in diameter, depending on the strain. Its external layer is composed of a hard, dark brown, lipoproteinaceous chorion (Anderson et al., 1970). Osmotic withdrawal of water, dehydration by air, or anoxia causes the encysted embryo to enter a state of diapause or cryptobiosis, during which the organism shows little or no sign of life. The cryptobiotic state of the cyst is remarkable in its ability to withstand such environmental extremes as essentially complete desiccation, temperatures over 100°C and near absolute zero, high energy radiation and a variety of organic solvents (Clegg, 1974). Yet only water and oxygen are required to initiate the normal development of the embryo. This durable, easily hatched diapause state makes *Artemia* cysts a convenient, constantly accessible source of live animals for the shrimp hatchery operator.

Today, there are many geographic strains of *Artemia*. More than 50 have been registered from countries throughout the world. Numerous commercial harvesters and distributors exist, selling brands of different qualities. The present cost of good quality cysts can range from $26 U.S. to $66 U.S. per kilogram. Each gram of cysts should yield 200,000 to 300,000 hatched nauplii.

The nutritional value of the newly hatched nauplii is thought to be influenced by their lipid level and fatty acid composition. This can be highly variable, depending on their geographic origin and batch. It is thought that the strain of *Artemia* and the diet and environment of the parents cause this variability.

Within 24 hours after being placed in seawater at 28°C, the chorion, or hard coating of the cyst, breaks (see Figure 25[a], Page 52). The embryo, still surrounded by the transparent hatching membrane is then released (see Figure 25[b], Page 52). The embryo can be seen moving within the membrane. Within a few hours, the nauplius breaks free of the hatching membrane and becomes free-swimming (see Figure 25[c], Page 52), using specially modified

antennae for locomotion and food filtering (Sorgeloos, 1979). The nauplii can live on yolk and stored reserves for up to 5 days (Olson, 1979), but its caloric and protein reserves diminish constantly during this time (Benijts et al., 1976).

It is important that the *Artemia* nauplii are harvested and fed to the shrimp larvae in their most nutritional form, i.e. as soon as possible after hatching. The common practice of storing the nauplii in aerated seawater, at room temperature (mostly in outdoor conditions) results in a continual decrease in the energy content of the nauplii. Techniques for the improvement of the nutritional value of the *Artemia* nauplii are being developed, but we will not discuss them here.

If it is not possible to use all of the *Artemia* immediately after hatching, they can be stored at 0°C to 4°C for 48 hours while viability remains above 90%. To prevent foaming, densities should not exceed 1,000 to 1,500 per ml (1×10^6 to 1.5×10^6 cysts per liter or approximately 5 grams of cysts per liter). Aeration should be supplied by means other than an airstone and an antifoaming agent can be used, if needed. A glass pipet connected to air tubing works well. The weight of the glass holds the pipet down and large bubbles are expelled.

In addition to the decrease in nutritional value (drop in dry weight and caloric content), there are also problems relating to the increasing in the size of the *Artemia* (they become too large for the earlier mysis stages to ingest) and their swimming rates (they become too fast for the larvae to catch). Compounding this is the fact that the *Artemia* will then grow faster than the shrimp and consume algae and produce metabolites. The hatchery operator must then accommodate valueless *Artemia*, which he/she finds difficult to separate from the shrimp.

There are several problems associated with the chorion, or outer shell, of the *Artemia* cysts. They may contaminate the medium and/or *Artemia* and shrimp larvae with bacteria. Disinfection of the cysts will overcome this problem (see Exercise IV, Objective 2, Page 54 and Exercise V, Page 59).

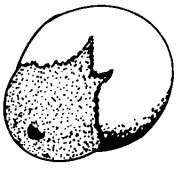
The shells themselves can cause certain problems. It is sometimes very difficult, especially with certain brands of *Artemia*, to separate the nauplii from the discarded shells. The addition of salt to the incubation container may help this separation and will not harm the Instar I nauplii.

The empty shells may carry bacteria, particularly if the cysts are not disinfected before incubation. They can also clog the gut of the shrimp larvae. The *Artemia* nauplii expends energy and thus reserves, which could otherwise be passed on to the shrimp larvae, in breaking out of the shell. The technique of decapsulation can be used to overcome all of these problems. (See Exercise V, Page 59). Here, the offending shells are removed chemi-

cally before the cysts are incubated. In addition to overcoming all the above problems, the decapsulated cysts are immobile and are smaller than the hatched nauplii. They can therefore be fed to shrimp larvae at an earlier stage. In this case, they should be kept in suspension through aeration, as the cysts would otherwise sink. A typical *Artemia* hatching stand arrangement can be seen in Figure 26 on Page 53.

Figure 25. Stages of *Artemia* Development
(from Treece & Wohlshlag, 1987)

a.

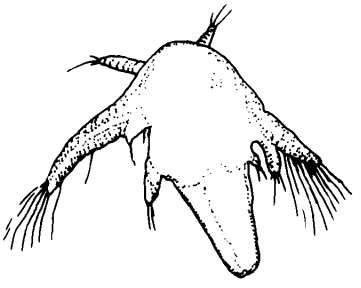


300μm
Pre-nauplius in E-1 stage

b.

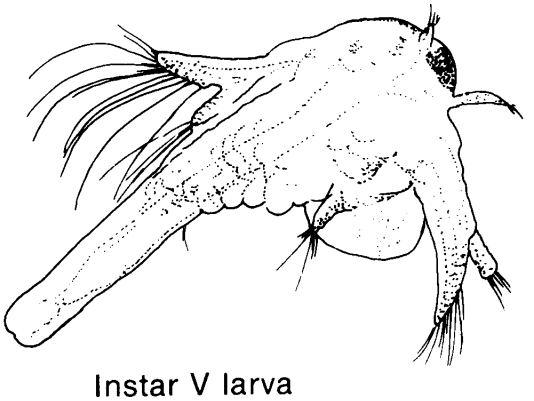
Pre-nauplius in E-2 stage

c.



Freshly hatched instar I nauplius

d.



Instar V larva

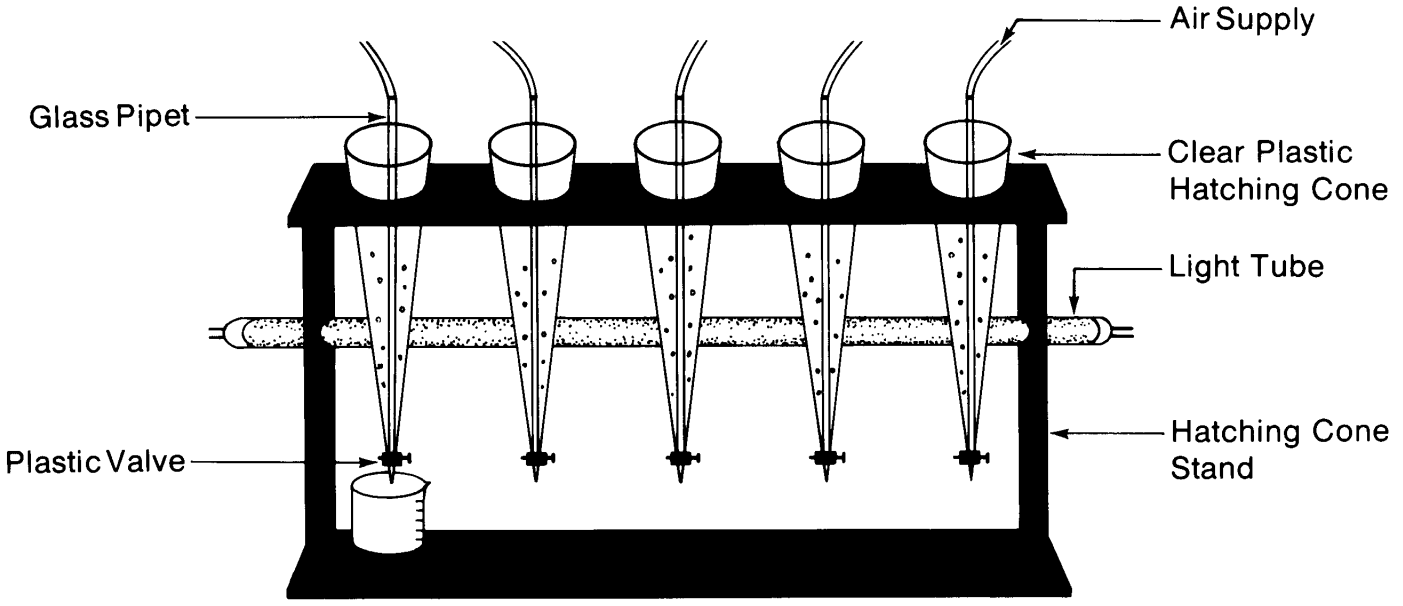


Figure 26. *Artemia* Hatching Cones
(from Treece & Wohlshlag, 1987)

EXERCISE IV. PREPARATION AND USE OF ARTEMIA

OBJECTIVES:

1. To determine the weight of *Artemia* cysts required to feed the larvae in a tank of known volume.
2. To hydrate, disinfect, incubate and separate *Artemia*.
3. To count the volume of *Artemia* nauplii per milliliter in the new batch.
4. To calculate the number of *Artemia* nauplii remaining in the LRT from a previous feeding.
5. To calculate the number of *Artemia* nauplii required by the shrimp larvae and to transfer them to the LRT.

MATERIALS AND EQUIPMENT

Artemia cysts.

Two 250 ml beakers.

Distilled water.

Household bleach.

1-liter Imhoff cone (see Figure 26 on Page 53).

Compressed air supply.

Seawater or equivalent (salinity approximately 32 ppt.).

Siphon tube, approximately 4'0" long, or a valve at the bottom of the cone (see Figure 26 on Page 53).

1 ml pipet.

10 ml pipet.

METHODS:

(1) Determining the amount of *Artemia* cysts required: The shrimp larvae are first staged. Their predicted daily *Artemia* requirements per ml are then found in Table II, Page 22. This feeding rate can be adjusted slightly, depending on the stocking density (number of larvae per liter) and the rate at which the *Artemia* are consumed. The total requirements are then calculated by multiplying the predicted requirements per ml by the total volume of the LRTs. We know that each gram of cysts contains approximately 200 to 300 thousand cysts and can safely allow a 50% hatch rate for our *Artemia* (experience with your specific brand will allow you to adjust these figures).

As an example, say that you find your larvae to be in the Mysis 2 substage. We know that in 24 hours time, when the *Artemia* have hatched, the larvae will be in the M3 substage. Table II, Page 22, shows that the shrimp larvae require 6 *Artemia* nauplii per ml.

You wish to feed 10 LRTs, each 16 liters in volume.

Your required hatched nauplii is therefore: 6 *x* 10 *x* 16,000 = 960,000 nauplii.

If you assume a 50% hatch rate, you require: 960,000 *x* 2 = 1,920,000 cysts.

Further, assume that one gram contains 250,000 cysts. Your required weight of cysts is therefore: 1,920,000/250 = 7.68, say 8 grams.

It is inadvisable to incubate more than 5 grams per liter of water, as this could cause foaming. We therefore choose two 1-liter Imhoff cones, using 4 grams per liter in each.

In the form of a formula:

$$\text{Weight of } \textit{Artemia} \text{ cysts required} = \frac{\text{Total vol. of all LRTs (in ml) } X \text{ No. of } \textit{Artemia} \text{ required per ml}}{\text{Percentage hatch rate } X \text{ No. of cysts per gram}}$$

(2) Disinfection: Soak the cysts for one hour in 80 mls of a 20 ppm. hypochlorite/tap water solution (approximately 50 mls solution per 5 grams of cysts). This could be achieved by adding 4 ml of household bleach to 10 liters (a little more than two and a half gallons) of water, resulting in enough solution for two pounds of cysts. To save time, this can be changed to soaking for twenty minutes in a 200 ppm. solution. The cysts should be aerated to ensure that all the cysts are exposed to the disinfectant. Wash the cysts with tap water in a 100–120 µm sieve. The incubation can now proceed.

Incubation: The 8 grams of disinfected cysts should be transferred to 1-liter Imhoff cones and filled with one liter of seawater, of approximately 32 ppt. salinity and temperature of 25°C, for 24 hours. Some hatcheries use diluted seawater of approximately 16 ppt. salinity, because it is thought that less energy is required for the nauplii to emerge from the cysts at the lower salinity. Constant light is supplied at an intensity of approximately 2,000 LUX, by a florescent tube. Vigorous aeration, supplied to the Imhoff cones through an air line, held down by a glass pipet attached to its terminal end, ensures that the cysts are kept in suspension and are not shaded from the light.

Separation: After 24 hours of incubation, most of the cysts will be hatched. The aeration should be removed. The pinkish-orange nauplii will be seen swimming in "clouds". The empty shells tend to float, while the full, unhatched cysts and some debris sink. If left undisturbed for 5 minutes nauplii will concentrate toward the bottom of the cone. The valve can be opened at the bottom of the Imhoff cone, first to remove debris and then to catch freshly hatched nauplii. A siphon can also be used to remove first the debris and then the nauplii from the bottom. The nauplii are siphoned into a graduated beaker. If necessary,

they can now be washed, using a 100–120 μm strainer and seawater, and placed back in the beaker.

(3) To count the *Artemia* hatched: The level of seawater in the beaker, holding the concentrated, freshly hatched nauplii, should be brought to some easy to use volume, such as 100 ml. The nauplii are mixed continuously, while a 1-ml sample is drawn off with a pipet and placed in another beaker. The contents of this beaker is increased to 100 mls with distilled water. The nauplii have thus been diluted to 1:100 of the original concentration. Lugol's stain should now be added to kill the *Artemia*. Stirring again, a sample is drawn into a 1-ml pipet. By holding this up to the light, the number of *Artemia* between two major graduations can be counted. This gives the concentration of nauplii in 0.1 ml of the dilution (H). Now, the number counted should be multiplied by ten, to get the number of *Artemia* in one ml, and multiplied by the dilution factor (100), to calculate the number of *Artemia* per ml in the original beaker. To get the total number of *Artemia* in the original beaker, multiply the concentration per ml by the volume of the beaker in mls. This results in the concentration of *Artemia* per milliliter of the original 100 ml beaker.

In the form of a formula:

Number of *Artemia*/ml concentrated in 100 ml beaker is (G) = H x 1,000

(See Figure 27 on Page 60).

(4) To calculate the number of *Artemia* remaining in the LRT: A similar procedure is followed to determine the number of *Artemia* which remains in the LRT from previous feedings. A sample of approximately 100 ml, is scooped from the LRT, into a beaker. The *Artemia* are killed by the addition of Lugol's stain. The same counting procedure and calculation is followed as for the above, except that no dilution is made.

Therefore, the formula becomes:

Concentration of *Artemia* per ml (I) = number per 0.1 ml (J) x 10

Other methods of subsampling may also be used. These include the use of a Hensen-Stemple pipet or some other automatic pipet system with a large intake so as not to limit the uptake of animals (See Figure 28 on Page 60).

(5) To calculate the number of *Artemia* to be fed: The substage of the shrimp larvae is determined (see Figures 9 and 10 on Pages 17 and 18) and is used to decide the total quantity of *Artemia* required per ml of the LRT (K) (see Table II on Page 22). By subtracting the number of *Artemia* per ml in the tank from this quantity, we find the net quantity per ml to be added; i.e. quantity of *Artemia* to be added (L) = K - J. When multiplied by the volume of the tank in mls, we find the total number required per tank (M). This should be divided

by the number of nauplii per ml in our fresh batch of *Artemia* (G), to ascertain the volume in ml thereof to be transferred to the LRT.

It may be easier to calculate with the following formula:

$$V = \frac{(K - H \times 1{,}000) \times M}{10 \times J}$$

Where: V is the volume of the new batch of *Artemia* to be transferred to the LRT;

H is the number of nauplii counted in the 0.1 ml sample of the 100:1 dilution of the fresh batch;

J is the number of nauplii counted in the 0.1 ml sample, taken from the LRT;

K is the number of nauplii per ml required by the shrimp larvae in the LRT (see Table II, Page 22), and

M is the volume (in ml) of the LRT.

The required volume of the newly hatched *Artemia* can now be drawn from the beaker, while being thoroughly agitated, and fed to the shrimp larvae. For our purpose, a 10-ml pipette should be used.

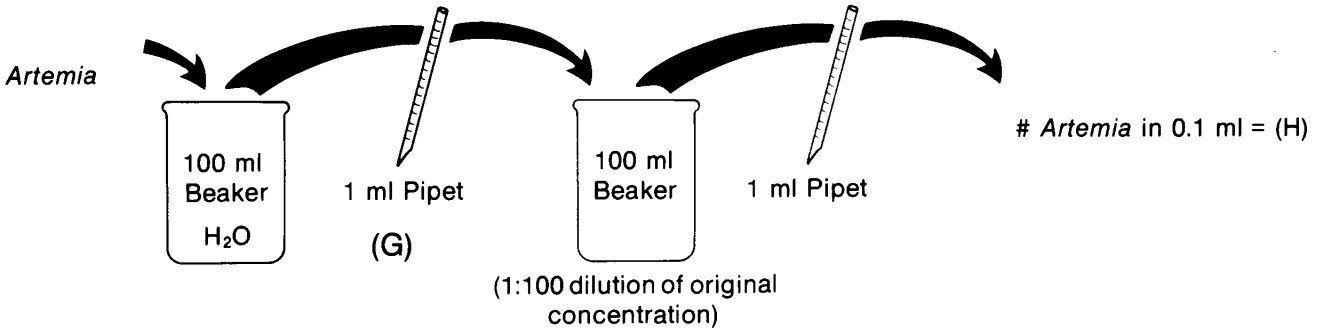
A general formula for *Artemia* calculations follows (from Wilkenfeld et al., 1986, unpublished manuscript):

Desired # *Artemia* (nauplii/ml) – **Remaining # *Artemia* in LRT (nauplii/ml)** *X* **LRT volume (ml)**

= **# of *Artemia* added to LRT**

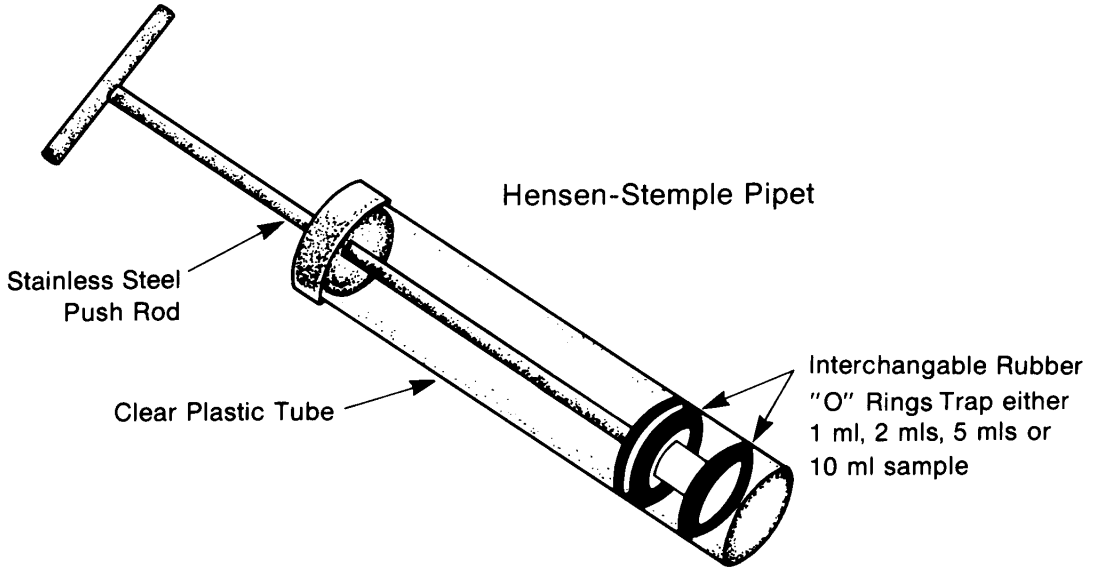
Figure 27

Counting *Artemia* when concentrated



$$(G) = (H) \times 1{,}000$$

Figure 28



EXERCISE V. ARTEMIA DECAPSULATION

OBJECTIVE:

To decapsulate a batch of *Artemia* cysts to be hatched.

MATERIALS AND EQUIPMENT:

Artemia cysts.

Distilled water.

Seawater or equivalent (salinity approximately 32 ppt.).

Household bleach.

Sodium hydroxide (NaOH).

0.1 N hydrochloric acid.

Approximately 1-liter flask.

100 - 120 µm sieve.

Round glass pie dish.

Magnetic stirrer, with plastic coated stirrer.

Chemical scale.

METHODS:

Decapsulation:

(1) Weigh the cysts and place them in a flask. Hydrate them with distilled or tap water for one hour.

(2) Pour the cysts into a 100–120 µm sieve, rinse under tap water and allow to drain.

(3) Seawater (10 ml per gram of cysts) and the plastic-coated stirrer should be placed inside the pie dish on the magnetic stirrer. When large volumes of cysts are to be decapsulated, NaOH and ice should be used to prevent heat build-up. NaOH is added to the seawater at a rate of 150 mg per gram of cysts and allowed to dissolve. The ice is placed in the solution to keep the temperature below 30°C. The cysts are then scraped from the sieve, after rinsing and the last few are washed into the dish with the bleach, at 5 ml of bleach per gram of cysts. With the addition of the bleach, the reaction (decapsulation) begins.

(4) *Slowly* turn up the speed of the stirrer and run it as fast as possible, without splashing.

(5) Watch the solution carefully. A white foam layer will develop and the solution will change from brown to light orange. This should take approximately 6 minutes. When no more color change is seen, the decapsulation process is complete.

(6) Immediately drain the pie dish through a 100–120 μm sieve, over the sink, and wash the cysts thoroughly with tap water. The washing should continue for about 10 minutes until no chlorine smell can be detected.

(7) Scrape the cysts into a beaker and pour in enough 0.1 N HCl to wash the cysts, for *no more than* 30 seconds. This neutralizes any remaining chlorine.

(8) Pour the cysts into the sieve again and wash for 3 minutes.

The cysts are now ready for incubation. This procedure should be carried out as in Exercise IV on Page 55 (incubation).

WARNING: GREAT CARE SHOULD BE TAKEN WITH BOTH THE BLEACH AND THE HYDROCHLORIC ACID, WHICH ARE CAUSTIC AND CAN CAUSE INJURY, PARTICULARLY TO THE EYES.

VIII. LARVAL DISEASES
(Liao, 1984)

In the initial period of the development of the prawn industry, unsuitable and insufficient food, resulting in substandard nutrition and starvation, were major causes of larval mortality. Occasionally, non-lethal or low-mortality diseases caused by protozoan infections occurred, but no serious larval diseases or high mortality were encountered, hence no papers were written on the subject. In contrast, since penaeid culture has become popular, and profitable in recent years, hatcheries are often overcrowded with larvae and this is generally accompanied by the occurrence of diseases. White-turbia midgut gland disease has been reported in *P. japonicus* (Shigueno, 1985), as well as *Lagenidium* infection in all penaeids (Couch, 1942; Cook,1971; Lightner and Fontaine, 1973; Lightner 1977; Lightner and Redman, 1981; Lightner, 1983). Baculovirus penaei (BP) disease in *P. aztecus*, *P. duorarum*, *P. setiferus*, *P. stylirostris* and *P. vannamei* (Laramore, 1977; Couch, 1978; Overstreet, 1978), and recently also baculoviral midgut gland necrosis (BMN) in *P. japonicus* (Sano et al., 1981), monodon baculovirus (MBV) disease in *P. monodon* (Lightner and Redman, 1981; Lightner, 1983), and finally infectious hypodermal and hematopoietic necrosis (IHHN) in *P. stylirostris* and *P. monodon* (Lightner, 1983), all have proven to be a serious threat to hatchery business. It is however not yet known if MBV is an important disease. Table III on Pages 63 and 64 (from Fox, 1987) summarizes the major diseases in the larval and postlarval stages of penaeids and the corresponding treatments.

It is commonly believed that diseases may increase in variety and occurrence as time passes, especially with respect to the virus-caused diseases. For the present, only four vital diseases are identified in penaeid larvae, but it is likely that more will be found. The ultimate concern is obviously for the prevention of diseases and how to reduce the devastating effect on larvae so that great losses can be avoided. MBV disease shows its lethal effect only when combined with the serious symptoms of other diseases. It is true that by providing MBV-infected larvae with a suitable environment and food they are better protected from other diseases and hence can attain normal growth. For the present, MBV is known to exist in Taiwan, Indonesia and the Philippines, but the extent of its range in other areas is unknown. Being an enzootic virus, MBV should be eradicated. To avoid further spreading, strict quarantines and burning of the infected larvae should be carried out (Lightner, et al., 1983).

In summary, the concept that prevention is more important and effective than cure in controlling a disease is absolutely accurate. Reducing stress due to crowding and to execute quarantines are as necessary as the continuing research on viruses and determining the etiology of other diseases.

It is recommended that you identify the disease by microscopic examination and compare it to one of the publications which have figures or photographs of the diseases (such as Johnson's **Handbook of Shrimp Diseases,** 1978 or Lightner's **Diseases of Penaeid Shrimp,** CRC, 1983). Then when identified, use either Lightner's recommended treatment or one of the treatments listed in Table III on Page 63.

An example of a suggested larval culture feeding regime and disease preventive schedule can be seen in Table IV on Page 65. This regime and schedule has proven success in a commercial *P. monodon* hatchery in Indonesia, presently operating as a model hatchery on the Island of Java.

Table III. Diseases Found in the Developmental Stages of Penaeid Larvae and Their Control Methods (Fox, 1987)

Disease	Affected Parts	Symptoms	Treatment		Life Stages Affected*	References
Bacteria						
Bacterial necrosis	Appendages	Appearing as localized necrosis or discoloration on any appendage, causing high mortality of zoea and mysis stages, affects post-larva to a lesser extent.	Furnace Erythromycin Achromycin	1.1 ppm 1.5 ppm 1.2 ppm	Z1 M1 PL	Tareen, 1982 Lightner
Vibrio infection	Hemolymph, midgut gland	Initial stages of one form, some larvae will show yellow-vermillion and red color permeating entire nervous system. Another form exhibits "White-turbid liver"; where the midgut gland of the larvae becomes generally white-turbid. Turbidity becomes more apparent and well-defined as the disease progresses.	Furazolidone Teramycin Furnace	2.0 ppm 450 mg/kg biomass 1.3 ppm	PL	Nickelson and Vanderzant, 1973 Lewis, 1973 Shigueno, 1975 Lightner, 1977 Johnson, 1978 Cipriani, et al. 1980
Filament-ous bacteria	Gills, pleopods	Commonly found attached to the gill filaments and the pleopods, turning blackish when bacteria mix with dirt. If severly affected, the respiratory function of the gill suffers damage.	Cutrine plus Malachite green Potassium permanganate Cuprous chlorine	0.5 ppm 10ppm 8.5 ppm 1.0 ppm	PL	Delves-Broughton and Poupard, 1976 Streenbergen and and Schapiro, 1976. Johnson, 1978 Solangi, et al., 1979 Tareen, 1982 Lightner, 1983
Shell disease	Exoskeleton, muscles	If infected by chitinoverous bacteria, the exoskeleton will display eroded blackened areas. Also bacteria can rapidly enter the body through surface breaks to cause internal damage.	Malachite green and Formalin combined	0.9 ppm 22 ppm	PL	Cook, 1973 Delves-Broughton and Poupard, 1976 Johnson, 1978 Tareen, 1982 Lightner, 1983
Black gill disease	Gills	In initial stages, gill color turns dull orange-yellow or light brown, when advanced, the area darkens until it is finally black.	Malachite green Methylene blue	3.0 ppm 8-10 ppm	PL	Shigueno, 1975 Tareen, 1982
Fungi						
Lagenidium infection	Body cavity, appendages	Only thin-cuticled prawn can be infected thus larval prawn are highly sensitive, The hyphae appear inside the body of zoea and continue into mysis stage, resulting in massive muscle destruction and heavy mortality of zoea and mysis.	Treflan Malachite green	0.1 ppm 0.01 ppm	Z1	Hubschaman and Schmitt, 1969 Lightner and Fontaine, 1973. Lightner. 1977 Johnson, 1978 1980 Tareen, 1982 Lightner, 1983

Table III. Diseases Found in the Developmental Stages of Penaeid Larvae and Their Control Methods (cont.)

Disease	Affected Parts	Symptoms	Treatment		Life Stages Affected*	References
Ectocommensal protozoea						
Ciliate infection (*Zoothamnium* sp. *Epistylis* sp.)	Gills, eyes Exoskeleton	Heavy infestation by *Zoothamnium* sp. on gills and eyes of larval prawn results in high mortality. *Espistylis* sp. seems to prefer exoskeleton as attacment site and is less harmful. When abundant on gill surface, both can cause hypoxia and death. Additionally, their abundant presence on general body surface of larvae may interfere with locomotion, feeding, molting, etc. Parasite burden increases until ecdysis provides relief.	Malachite green and Formalin combined Quinacrine hydrochloride Chloramine- Methylene blue Saponin 10%	1.0 ppm 25 ppm 0.8 ppm 5.5 ppm 8.0 ppm 5.0 ppm	Z	Johnson, et al., 1973 Overstreet, 1973 Delves-Broughton and Poupard, 1976 Lightner, 1977 Liao, et al., 1977 Johnson, 1978 Lightner, et al., 1980 Tareen, 1982 Lightner, 1983
Viruses						
Penaeid baculo-viruses (8P, M8V, 8MN).	Hepatopan-creas, Anterior midgut	Penaeid baculoviruses infect epithelial cells of the hepatopancreas and, less commonly, anterior midgut, causing high mortality in the post-larval stage.			PL	Johnson, 1978 Sano, et al., 1981 Lightner, 1983 Lightner, et al., 1983 Couch, 1974
Infectious hypodermal and hemato-poietic necrosis (IHHN)	Hypodermin, Hematopoietic organs	Prawn dying from acute IHHN show massive destruction of cuticular hypodermis and often of the hematopoietic organs, of glial cells in the nerve cord, and of loose connective tissues such as the subcutis and gut serosa. Only prawn within a size range of 0.005-1.0 g have been observed to have these epizootics, resulting in massive mortalities (often 80 to 90% within 2 weeks) of onset)			PL	Lightner, 1983
Miscellaneous Diseases						
Abnormal nauplii	Appendages	Occurs as a result of poor quality of spawner.			N	Tareen, 1982
Amoebasis of larvae	Subcutis, muscle	Invasion of muscles and subcuticular tissues located in the abdomen, cephalothorax, antenna, and eye stalks, by unclassified amoeba.			Z	Laramore and Barkate, 1979 Lightner, 1983
Larval encrusta-	Exoskeleton	Brown to black encrusted deposits which contained iron salts affect larval penaeids			Z1 M1 PL	Lightner, 1983

*N=nauplius, Z=zoea, M=Mysis, PL=post-larva

Table IV
Suggested Larval Culture Sequences, Postlarval Feeding Regime and Disease Preventive Schedule for a Commercial *P. monodon* Hatchery in Jepara, Indonesia.

				Feeding					Treatments	
Day	Tank	Vol. (T)	Stage	*Chaet.* (cells/ml)	*Tetra.*	*Art.* (#/ml)	Pellet % Body wt./day	Water % exch.	Treflan (ml/T) 5 ppm Sol.	Chlor** ppm
0	ST	0.8	E-N	-	-	-	-	200	-	-
1	LRT	1	N	-	-	-	-	0	-	-
2	LRT	1	N6-Z1	50,000	-	-	-	0	20	-
3	LRT	2	Z1	75,000	-	-	-	0	30	2
4	LRT	3	Z1-Z2	100,000	20,000	-	-	0	30	-
5	LRT	3.2	Z2	100,000	20,000	-	-	0	40	-
6	LRT	3.5	Z3	100,000	20,000	-	-	0	40	-
7	LRT	3.8	Z3-M1	50,000	20,000	1	-	50	40	-
8	LRT	3.8	M1	50,000	20,000	1	-	50	40	-
9	LRT	3.8	M2	Trace	20,000	3	-	50	50	4
10	LRT	3.8	M3	Trace	20,000	6	-	50	50	-
11	LRT	3.8	M3-PL	-	20,000	6	-	50	50	6
12	LRT	3.8	PL1	-	20,000	6	-	50	50	-
13	LRT	3.8	PL2	-	Trace	6	-	50	50	-
14	LRT	3.8	PL3	-	Trace	6	-	50	50	-
15	LRT	3.8	PL4	-	-	6	-	Flush	-	-
15	RWY	7-12	PL4	-	-	6	-	100	50	*
16	RWY	7-12	PL5	Trace	-	6	-	100	50*	*
17	RWY	7-12	PL6	Trace	-	5	100%	100	50*	*
18	RWY	7-12	PL7	Trace	-	4	200%	100	50*	*
19	RWY	7-12	PL8	Trace	-	3	200%	100	50*	*
20	RWY	7-12	PL9	Trace	-	2	200%	100	50*	*
21	RWY	7-12	PL10	-	-	1	200%	100	50*	*
22	RWY	7-12	PL11	-	-	0	200%	100	50*	*
23	RWY	7-12	PL12	-	-	0	200%	100	50*	*
24	RWY	7-12	PL13	-	-	0	200%	100	50*	*
25	RWY	7-12	PL14	-	-	0	200%	100	50*	*
26	RWY	7-12	PL15	-	-	0	200%	100	50*	*
27	RWY	7-12	PL16	-	-	0	200%	100	50*	*
28	RWY	7-12	PL17	-	-	0	200%	100	50*	*
29	RWY	7-12	PL18	-	-	0	200%	100	50*	*

*Treat if Needed
Also use Malachite Green (1ppm) if *Zoothamnium* is noted.

** Chlor = Chloramphenicol (antibiotic)
RWY = Raceway
LRT = Larval Rearing Tank
Chaet. = *Chaetoceros*
Tetra = *Tetraselmis*

TECHNICAL SHEET I

THE USE OF THE HEMACYTOMETER

(Note: Not all hemacytometers are the same. Only one variety is described here.)

INTRODUCTION: The hemacytometer consists of two parts. The major element is formed from a slab of thermal and shock resistant glass. Into this, an H-shaped trough has been cut, forming two raised counting areas. Raised shoulders on either side of the H are precision milled to exactly 0.1 mm above the counting area. The cover glass, a 0.4 mm highly polished piece of glass, rests on the shoulders, forming the top of the counting chamber.

The counting areas are covered with a thin metallic film, which gives them a slightly darkened appearance, under the microscope. Into this film, lines are scribed with great precision (see Figure 29, Page 68). The "tic-tac-toe" pattern has nine squares, each 1 x 1 mm. These are each divided into 25 smaller squares and the center square is further subdivided into 400, 0.05-mm, squares.

PREPARATION OF SAMPLE AND HEMACYTOMETER:

(1) Before starting, the hemacytometer and cover glass should be cleaned and dried. A piece of lens paper, wetted with distilled water, should be used.

(2) A sample of algae is obtained from the algae culture tank or from the larval rearing tank. This is placed in a clean test tube and, if necessary, 2–3 drops of Lugol's stain are added and the tube shaken, to kill and immobilize the cells.

(3) The tube is agitated again so as to thoroughly mix the cells in the medium. A clean eyedropper or pipet is used to remove approximately 0.5 ml of the mixture.

(4) A single drop is introduced to the V-groove on the side of the hemacytometer. This will be drawn under the cover glass, into the counting chamber, by capillary action. Care should be taken to *introduce just enough to fill the counting chamber.* If the troughs were filled and shoulders wetted, the cover glass would lift and the counting chamber would enlarge. Should this occur, the hemacytometer should be cleaned and the process started again.

(5) The cells will take about a minute to settle. The hemocytometer is now placed on the stage of the compound microscope. Using the lowest power of magnification, the microscope is focused (see Technical Sheet IV, Page 77).

(6) For accuracy, a minimum of 100 cells should be counted. Depending on the concentration of the cells, this might require counting the entire scribed counting area, counting just the four large corner squares or counting all or some of the center square. This should be done systematically, counting the same blocks each time, thus eliminating bias.

(7) The calculations should then be carried out as follows:

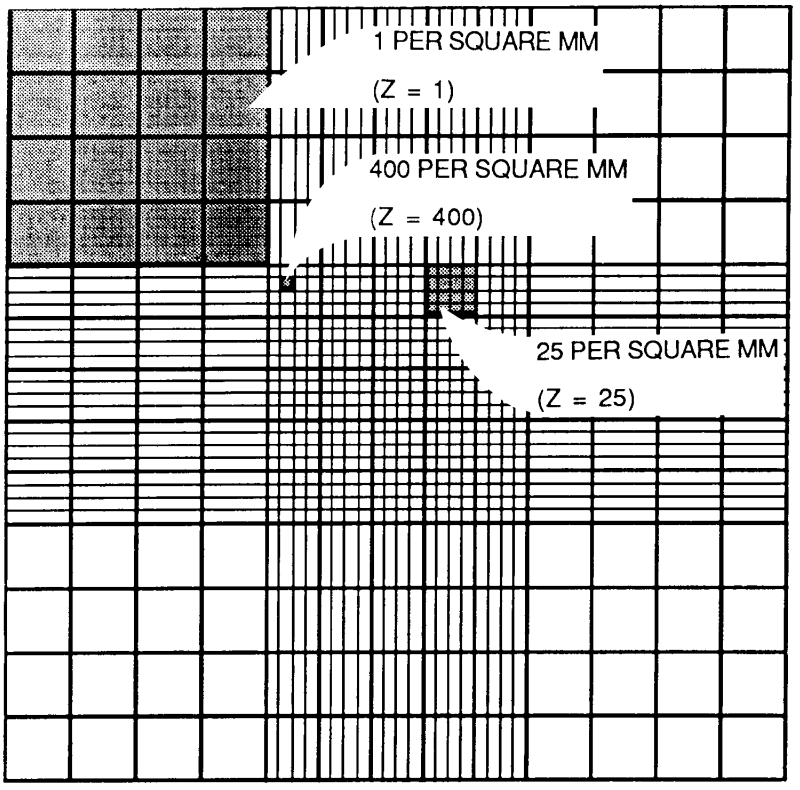
(a) The number of cells counted (X),

(b) should be divided by the number of square counted (Y),

(c) then multiplied by the number of squares per square mm (Z).

(d) Finally, this is multiplied by 10,000 to obtain the number of cells in each ml of medium (N).

I.e.; $N = \frac{X \times Z \times 10,000}{Y}$



MAGNIFICATION OF COUNTING AREA

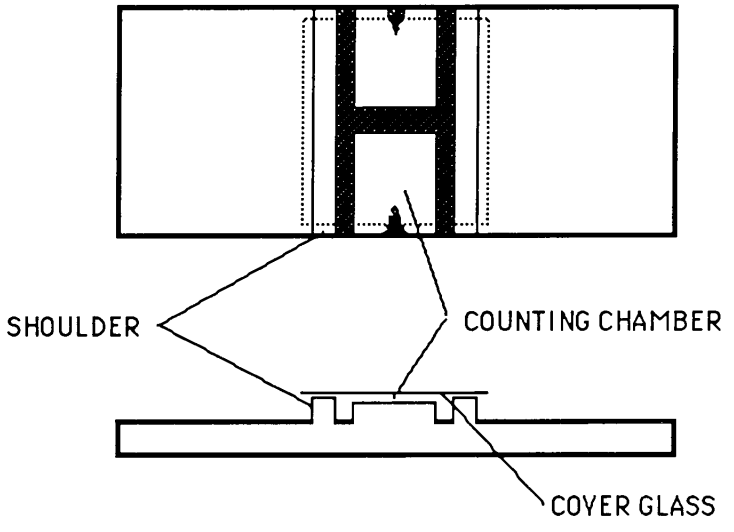


Figure 29. Hemacytometer

TECHNICAL SHEET II

STERILIZATION (HAMILTON, 1979)

It is not always necessary, nor even desirable to use sterile techniques. Not only is sterilization expensive and time consuming, but it can in some cases actually produce toxic substances, damage delicate apparatus and deteriorate chemicals in solution. In many instances, simple chemical cleanliness could be appropriate. Even when sterilization is required, chemical cleanliness forms the basis for most sterilization techniques.

Hamilton (1979) states the fundamental difference between *sterilization* and *disinfection*. "*Sterilization* is normally reserved for processes which ensure the total inactivation of all microbial life – bactericidal (note that these processes do not ensure the termination of all enzymatic life). However, *disinfection* is taken to mean the reduction of bacterial numbers to some arbitrary 'safe' or 'acceptable' level – bacteriostatic."

With specific applicability to glassware and axenic cultures of algae, not all the procedures listed may be necessary for every application, but they are as follows:

(1) Detergents: Domestic detergents may leave a residual film on glassware, but there are several satisfactory laboratory detergents available through scientific supply companies. "The detergent should remove organic matter below 100°C, and leave the glassware neutral after rinsing.

(a) Rinse the glassware in a stream of warm tap water immediately after use.

(b) Soak overnight in a detergent solution prepared in distilled water and contained in a stainless steel receptacle. (All visible residual should be scrubbed from the glassware).

(c) Heat or boil gently for approximately one hour, rinse in tap water followed by several rinses in distilled water.

(d) Invert to dry (never use a pegboard) and then cover.

(2) Acids: Glassware which fails to respond to detergent cleaning may be soaked in concentrated H_2SO_4 saturated with $NaNO_3$ or in hot HNO_3 (in a fume hood) followed by thorough rinsing with distilled water. Chromate cleaning solutions should never be used, as the chromium ion, which is quite toxic, adsorbs into glass and is difficult to remove.

(3) Baking: Small amounts of organic matter can be destroyed by baking the glassware at 350°C–400°C for 3–4 hrs. After the baking exercise, care must be taken to prevent the ingress of dust and other airborne contamination. Dirty fingers are a common source of contamination.

There are two types of disinfectants; non-volatile and volatile.

Non-volatile disinfectants: These, it is suggested, should be used only for the reduction of the microbial load on surfaces and instruments such as on bench tops, etc. Several chemicals which are commonly used are: Lysol®, chlorine (hypochlorite), formaldehyde, mercurial compounds and alcohol. Care should be taken in the choice and use of these agents. Some are virtually ineffective or take 24 hours to work. Lysol® leaves an oily residue, can contaminate an autoclave and can burn the skin in its concentrated form. Hypochlorite preparations and acidified alcohol may corrode metal instruments. Hypochlorite and formaldehyde can be serious health hazards. Mercurial compounds are difficult to remove from glassware, its effect on humans is unknown and mercuric chloride is deadly to fish.

A method of sterilization commonly used in the industry is "chlorination." Chlorox® bleach (5.25% activity) is mixed in seawater at 2.5 ppm. and allowed to sit for 24 hours. A liberal amount of air is then bubbled through the water to drive out the remaining chlorine. While sodium thiosulphate can be used to neutralize the chlorine, some hatcheries have reported negative effects therefrom, and its use is not recommended.

MOIST HEAT

This is the most widely used method of producing sterility. It is applicable to all medium and materials which will withstand a temperature of 100°C–120°C.

(a) Boiling Water Bath: Fill the bath with distilled water and raise to 100°C. Place medium or instruments on the bath, allow them to come to temperature, then begin timing and allow the treatment to proceed 5–10 minutes. This will ensure the death of all vegetative cells but will not affect spores. This method should be used only in emergencies. Its efficiency may be increased by the use of 2% (weight-to-volume) solution of sodium carbonate instead of distilled water, but this solution may corrode instruments.

(b) Commercial Steamer: This may be used in two ways, either to produce a single exposure for 90 minutes, or to provide intermittent exposures of 30-minute duration on three successive days. The latter process kills the vegetative cells produced by spores which germinate in the intervals between treatments. In order to ensure that medium attains temperature rapidly it is strongly recommended that medium volumes should not exceed 500 ml. If intermittent exposures are used, the containers must be cooled to room temperature for at least 18 hours between successive steam treatments. An autoclave heated to 100°C (no build-up of pressure) may be used as a steamer.

(c) Pasteurization: As with the intermittent steamer exposures, this technique aims to kill spores after they have incubated between heatings. The medium is heated, directly or with an immersion heater, to 73°C and held at that temperature for 10–15 minutes. It is allowed to cool to room temperature for 18 hours before the heating process is repeated. This method is easy and inexpensive. It causes less damage to vitamins in the medium and precipitation of phosphates than do methods which rely on high temperatures.

(d) Autoclave or Pressure Cooker: (For specific information on the use of the autoclave, see Technical Sheet III, Page 74).

Temperatures of 121°C or more will destroy bacterial endospores (the form of life which is most resistant to heat), if exposed for the correct length of time. The highest temperature that can be attained by steam at atmospheric pressure is 100°C. However, when under an added pressure of 15 lbs./square inch, steam can attain a temperature of 121°C. It is on this principle that the autoclave works.

Knowing this will help us to understand some important points in using the autoclave. Firstly, it is the steam which conducts the heat to the objects and liquids to be sterilized. In order to ensure sterility the steam must penetrate the materials. Therefore, instruments should be wrapped in materials which permit easy access of steam. Unwaxed Kraft® paper is considered the material of choice. Aluminium foil is in common use but it should be noted that it is quite impermeable to steam except by reason of poor wrapping procedures. Its use is, therefore, not recommended.

Secondly, as steam gets hotter, it becomes drier. At temperatures above 121°C exposure times must be increased to that of hot-air sterilization (1 hour at 170°C). Not only will excessively heated steam lose some of its efficiency, but it may increase damage to equipment and solutions to be autoclaved.

It is important to ensure that all air is expelled from the chamber before the exhaust valve is closed and pressure is allowed to build. If this is not done, the air and steam will stratify and sterilization will not be attained. The removal of air is accomplished automatically in commercial autoclaves. However, if a pressure cooker is being used, do not close the exhaust valve until a steady stream of pure steam is observed.

Time is required for the steam to penetrate and heat the materials to sterilizing temperatures. This will primarily depend on the individual volumes of medium to be treated. An autoclave may be purged of air and its gauges may indicate that it has reached operating temperature and pressure without the interior of the load itself being warm to the touch. Even when temperatures reach 121°C, the bacteria are not killed at once. Normally, it takes 11–12 minutes to kill the endospores of thermophilic bacteria. A volume of 100 ml

requires 15 minutes to reach sterility while 2 L requires 20 minutes and 5 L requires 35 minutes.

Autoclaves or pressure cookers may cause precipitation in some media, notably those prepared with natural or artificial seawater. This precipitate will contain almost all the phosphate originally present in the media. The precipitates may be reduced by the addition of sterile phosphate, trace metal and silicate solutions after autoclaving. In addition, some reduction is observed if the media is autoclaved in small lots.

Care should be taken with certain materials, including disinfectants (particularly formalin and Lysol®), plastics, cheesecloth, etc., which may become toxic when autoclaved. They may also contaminate the autoclave for future use.

Autoclaving is the most effective method of sterilization and the most commonly used in research facilities, but the equipment is expensive. A pressure cooker size autoclave costs approximately $300, while a carboy size autoclave costs approximately $3,000.

(e) Dry heat: Sterilization by dry heat requires the use of temperatures which are higher than those employed during the moist heat methods. Therefore its use is usually restricted to heat stable materials such as glassware.

Hot-air sterilization has the added advantage that if a high enough temperature is employed, there is a complete destruction of most organic matter. This is of benefit to those employing bioassay procedures or attempting to culture forms which are particularly sensitive to trace organic contaminants.

Bunsen burner, propane torch, alcohol lamp or torch may be used to heat inoculation needles or loops to incandescence. It may also be used for forceps and other metal instruments which will not be damaged by heat. As alcohol lamps burn with a cooler flame than that of a Bunsen burner, care must be taken to ensure that enough heat is obtained. Dipping in alcohol before flaming is frequently employed for instruments which would be damaged by red heat. This method does not necessarily ensure sterility. Flaming causes boiling in the initial stages resulting in splattering of material carried on the inoculating loop or needle. This splattering can cause aerosol formations which contain living cells. Delicate or sharp instruments as well as those constructed of materials which have differing coefficients of expansion may be damaged by dry heat sterilization.

Hot-air ovens may be used for sterilization of materials which can withstand temperatures of 160°C. The dry materials should be loosely packed into the oven and held at 160°C for 2 hours after it has reached that temperature. Temperatures of up to 400°C are recommended for baking glassware. Pipettes and tubes may be plugged with non-absorbent cotton wool before sterilization and with glasswool for baking.

FILTRATION

Here we deal with the filtration of small volumes of media in the laboratory. The filtration of the raw seawater for later use in the laboratory is dealt with in Chapter II.

Filtration is used for the production of sterile as well as particle-free solutions which might be affected by heat treatment. It is also used to sterilize air supplies for cultures and for the production of particle-free air for clean rooms.

Several types of filters are available for the filtration of liquids. Ultra-fine sintered glass filters are expensive, clog easily and are difficult to clean. Disposable membrane filters, including plastics, are more convenient, but some may leach impurities into the media. All filter equipment must itself be sterilized (by gas or autoclave) before use. Pressure or vacuum may be required to push or pull the medium through the filters. Filtration is generally slow, and expensive, but can be used for small volumes of media which would deteriorate if heated.

Air and CO_2 supplied to axenic cultures is usually first passed through a cotton filter. Filters which become damp from water vapor, may provide a troublesome source of contamination. They can be kept dry by gently heating with insulated Briskeat tape wrapped around the filter tubes.

RADIATION

Ultraviolet radiation finds wide application in the laboratory, particularly in the disinfection of solid surfaces. Its action is uncertain, especially when penetration is required, but UV is thought to break down DNA within the cells. The accepted dose is based upon the assumption that the initial concentration of bacteria is quite low and should be determined for each individual situation.

Ultraviolet radiation is frequently used to disinfect the top surfaces of tables in culture transfer rooms. "Ultraviolet filters" (lamps) are also available for the sterilization of fluids. For any particular filter, the dosage depends on the flow rate. Fluids should first be filtered through a fine filter (1.0 micron), so as to eliminate particles which could shield the bacteria against the radiation.

Great care should be taken with the use of this radiation. It will not only inflict severe damage to the naked eye, but will also burn the skin.

TECHNICAL SHEET III

OPERATION OF THE AUTOCLAVE

(1) Time can be saved by switching the autoclave on, to heat while the load is being prepared. In the case of the pressure cooker, this just requires heating the water. Be sure that it does not become too hot to load. The more sophisticated autoclaves are able to build up temperature and pressure in a "pressure jacket", independently of the chamber.

(2) Flasks, etc. should not be overfilled and should be capped with paper or plugged with cotton. Smaller volume containers require less time to autoclave. This will result in less damage to the medium and apparatus and less sedimentation of the sulfates. If rubber stoppers or screw caps must be used, they should be of autoclavable material. So as to allow steam to penetrate and to prevent containers from bursting, caps or stoppers should be set in place very loosely.

(3) Glassware, etc. should be packed loosely into the autoclave, allowing for circulation of the steam.

(4) The door/lid should be shut and "bolted." Most automatic autoclaves will not allow the door to be unbolted before the sterilizing cycle is complete.

NOTE: THE AUTOCLAVE WILL OPERATE UNDER HIGH TEMPERATURE AND PRESSURE. CARE SHOULD BE TAKEN TO ENSURE THAT THE TEMPERATURE AND PRESSURE HAVE DISSIPATED BEFORE THE DOOR IS UNBOLTED AND OPENED.

(5) The air in the chamber should now be replaced with steam. In the case of a simple pressure cooker, the exhaust valve is left open, while heat is applied. For the autoclave, the exhaust valve and then the steam inlet valve are opened. In each case, steam should billow out of the exhaust before it is shut. If the autoclave has a thermometer on its outlet, this should read 100°C, before the exhaust is shut.

(6) The pressure and temperature should be closely monitored until they reach 15 lbs/square inch and 121°C respectively. Only when the thermometer has reached 121°C, should the timing start. Allow 15 minutes for equipment, tubes and small flasks and 45 minutes for carboys.

(7) When autoclaving is complete, the heating element is switched off or the incoming steam valve is closed. The pressure is then allowed to dissipate slowly. Rapid decrease of pressure may cause liquids to boil-up and wet or eject their stoppers, thus losing sterility when removed from the autoclave.

(8) The door should be unbolted and opened only after the pressure gauge indicates that the pressure in the autoclave is zero.

(9) It is best to unload the equipment immediately, so as to allow it to cool rapidly. This will minimize the deterioration of solvent and the build up of sedimentation.

Some modern autoclaves will automatically perform steps 5 through 8, after the controls have been set.

LABORATORY SEPARATE **BIOLOGY** **400**

From Abramoff-Thomson: *Laboratory Outlines in Biology–IV*
Formerly Separate No. 846

EXERCISE **1**

Light Microscopy

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The light microscope is capable of extending our ability to "see detail" by 1000 times, so that objects as small as 0.1 **micrometer** (μm) or 100 **nanometers** (nm) can be seen. The transmission electron microscope extends this viewing capability to objects as small as 0.5 nm in diameter, enabling us to see objects that are $\frac{1}{200{,}000}$th the size of those that can be seen by the human eye. Without microscopes, our understanding of the structure and function of cells and tissues would be severely limited.

The ability of the microscope to reveal the structure of small objects, however, is not so much a function of its ability to magnify as its ability to distinguish detail. Merely magnifying an object, without increasing the amount of detail seen is of little value to the observer. The ability to see detail is called **resolving power** and depends on the wavelength (λ) of light used and a value called the **numerical aperture** (NA), an important characteristic that determines how much light will enter the lens. In its simplest form, resolving power, or **resolution,** may be expressed by the formula

$$\text{RP} = \frac{\lambda}{2 \times \text{NA}}$$

λ = wavelength of light used
NA = numerical aperture

Under normal viewing conditions, resolution is *increased* by decreasing the wavelength of the light source. For example, if you use a green filter that permits a wavelength of 500 nm to pass through a microscope lens having a numerical aperture of 1, then the resolving power would be 500 nm/2 × 1 or 250 nm. This means that two objects that are 250 nm or farther apart would be seen as distinct objects; if closer than 250 nm, they would appear very fuzzy or as one object.

If you use blue light, or a blue filter that provides light at a wavelength of 400 nm and a lens having a NA of 1, the resolving power would be equal to 400 nm/2 × 1 or 200 nm. The two objects observed under these conditions could be 50 nm closer together and still be seen as separate objects.

Knowing the significance of the wavelength of light to the ability to distinguish detail, you can appreciate the role of electron microscopes and micro-

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scopes utilizing ultraviolet light in elucidating the structure and function relationships of cells and subcellular organelles.

A. PARTS OF A COMPOUND MICROSCOPE

Your microscope may have all or most of the features described below. Referring to Fig. 1-1, locate the following features of the microscope available in your laboratory.

1. Ocular Lens

The **oculars** are the lenses you look through. If there is only one ocular, you are using a **monocular** microscope; if there are two, it is a **binocular** microscope. In many binocular microscopes, the oculars can be adjusted to compensate for differences in distance between your eyes **(interpupillary adjustment).** One of the oculars may have a knurled adjustment mechanism for moving it in and out to compensate for focusing disabilities between each eye. Your instructor will describe how this is done. Oculars on different microscopes may have different magnifications. You may have to remove the ocular from its holder to determine its magnification. What is the magnification stamped on the housing of the oculars on your microscope?

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The ocular contains a series of several magnifying lenses and may also include an **ocular micrometer** (a scale for measuring objects) and a pointer (to point out objects to your instructor or other students).

2. Objective Lens

Attached to a rotating nosepiece, or turret, at the base of the body tube are a group of three or four **objectives.** Rotate the nosepiece and notice that a "click" is heard as each objective comes into position.

The magnifying lenses of the objectives focus light that comes from the specimen and passes it up the body tube and through the oculars.

Each objective has numbers stamped on it. One of these numbers identifies the magnification of the objective (e.g., 43 ×). What are the magnifications of each of the objectives on your microscope?

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The **total magnification** is calculated by multiplying the magnification of the ocular and objective lenses on the microscope being used. In Table 1-1 calculate the total magnification for each ocular/objective combination on your microscope.

TABLE 1-1
Calculation of total magnification for various ocular/objective combinations.

Ocular	×	Objective	=	Total magnification

Note: Objective lenses are usually named according to their magnifying power, as follows:

scanning power— 4 ×
low power— 10 ×
high or high dry power— 43 ×
oil immersion— 93 ×

A second set of numbers, usually given as a decimal, represents the numerical aperture for that lens; the abbreviation NA may precede the number. In Table 1-2 list the magnification and numerical aperture for each objective on your microscope.

TABLE 1-2
Numerical aperture and magnification for various objectives.

Magnification of objective	Numerical aperture (NA)

3. Body Tube

Light travels from the objectives through a series of magnifying lenses in the body tube to the ocular. In some microscopes, the body tube is straight. In

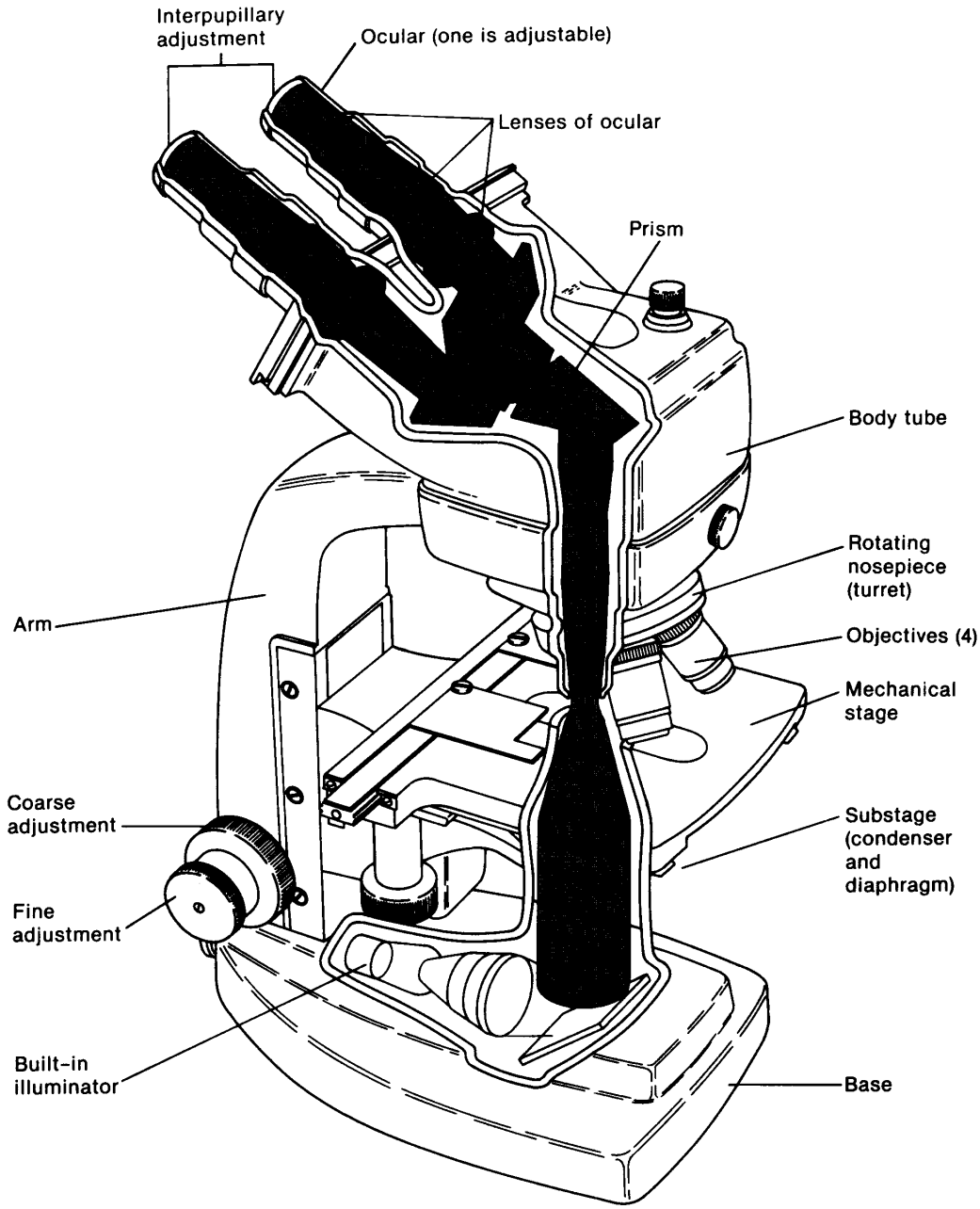

FIG. 1-1
Bausch and Lomb binocular microscope sectioned to show pathway of light from illuminator through various lenses and prisms.

others, the oculars are held at an angle, as in Fig. 1-1, and the body tube contains a prism that bends the light rays coming through the objectives so they will be transmitted through the oculars.

With some compound microscopes, loosening a lock screw allows you to rotate the body tube 180°. What is the advantage of being able to turn the body tube?

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4. Stage

The surface or platform on which the microscope slide is placed is the **stage.** Note the opening **(stage aperture)** in the center of the stage. On some micro-

scopes, the stage may be stationary and have clips to hold the slide in place. On other microscopes, the stage can be moved and is therefore called a **mechanical stage.** Movement is controlled by two knobs located on top, on the side, or on the bottom of the stage. Note the **horizontal** and **vertical scales** on the mechanical stage. What is the function of these scales?

How are slides held in position on a mechanical stage?

5. Substage

The area beneath the stage is the substage and may be occupied by one, or both, of the following:

a. Diaphragm

The diaphragm regulates the *amount* of light passing from the light source through the specimen and through the lens system of the microscope. By properly adjusting the diaphragm you provide better contrast with the surrounding medium, thus greatly improving your image of the specimen. The diaphragm may be either

1. An **annular** type of diaphragm consisting of a circular plate with holes of different diameters. This plate is rotated so that the various holes may be positioned in the light path to regulate the amount of light passing from the light source through the object under observation.

2. An **iris** type of diaphragm that consists of a series of overlapping thin metal plates. A lever projecting from the side of the diaphragm opens and closes these plates to regulate the amount of light entering the microscope.

What type of diaphragm does your microscope have?

b. Condenser

The condenser consists of a series of lenses that focus light onto the specimen. Movement of the condenser is regulated by a knob at its side, or a lever projecting from the condenser housing. By properly adjusting the condenser you greatly improve your observation of the specimen.

Attached to the bottom of the condenser may be a filter holder, which normally contains a blue filter. Why would you use a blue filter, instead of a green or red filter, when making microscopic observations? Indeed, why use a filter at all?

6. The Light Source

Your microscope may have an attached mirror or a built-in illuminator. If your microscope uses a mirror, one surface is usually concave and the other is flat. The flat side of the mirror is normally used with the scanning and low power objectives and the concave mirror with higher power objectives. The light source for the mirror is usually a lamp. Natural light may be used, but it is not preferred because the light's intensity will vary greatly, depending on the source of light in your laboratory.

In most compound microscopes, the illuminator is built into the base of the microscope and controlled by an on/off switch. You can control the amount of light entering the specimen by adjusting the diaphragm. You can also control the light intensity by adjusting a transformer attached to the illuminator, whose knob can be turned to regulate the voltage to the light bulb. Use low or medium transformer settings for most microscopic observations. You will need a higher setting when using the oil immersion lens. Why?

7. Focusing

You can focus your microscope by using the coarse and fine adjustment knobs that raise or lower either

the body tube or the stage, depending on the type of microscope you are using.

With the low-power objective in position, rotate the coarse adjustment knob one half turn clockwise. Do the same with the fine adjustment knob. Based upon your observations, why should you not use the coarse adjustment knob for focusing when the high-power objective or oil immersion objective is in position?

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8. Eyeglasses and Microscope Usage

Should you wear your eyeglasses when using a microscope? The answer to this question is qualified. If you are near- or farsighted, you need not wear your glasses for microscopic observations. The adjustments made in focusing the microscope will compensate for these eye problems. On the other hand, wear your glasses if you have astigmatism (a defect in the eye's refractive surface), since this problem is not corrected by the lenses of the microscope.

In either case, when using a monocular microscope, you should keep both eyes open, despite a tendency to close one eye. Eye strain will develop if you do this for any length of time.

B. PROPER USE OF MICROSCOPES

Before using your microscope, thoroughly clean the oculars and objectives using lens paper in a circular motion to prevent scratching. When using the microscope, keep eyelashes from touching the ocular. Oil from the lashes will adhere to the ocular lenses smearing them. When using salt solutions or other harsh chemicals to prepare wet mounts, thoroughly clean the oculars and objectives, stage, and microscope slides after use to prevent damage to the microscope.

Despite its sturdy appearance a microscope is a delicate, precision instrument. It should be handled carefully and with common sense. The following suggestions will help you avoid some common mishaps that occur when using a microscope.

1. To avoid dropping a microscope, banging it against a laboratory bench, or having the oculars fall out,

   a. Carry the microscope upright using both hands.

   b. Place the microscope away from the edge of the bench, particularly when not in use.

   c. Move power cords out of the way, so that you can't trip on them and pull the microscope or transformer down.

2. To avoid breaking a coverslip and/or microscope slide by an objective,

   a. First locate the specimen using the low-power objective, and then switch to the higher-power objectives.

   b. Never focus the high-power objective with the coarse adjustment knob and never use these lenses when examining thick specimens or whole mounts of specimens.

3. To avoid mechanical difficulties with various parts of a microscope,

   a. Never force microscope parts to work.

   b. When changing the bulb in the built-in illuminator, never force it, since it might shatter in your fingers.

   c. Never try to dismantle the microscope.

C. USING A COMPOUND MICROSCOPE

1. Focusing

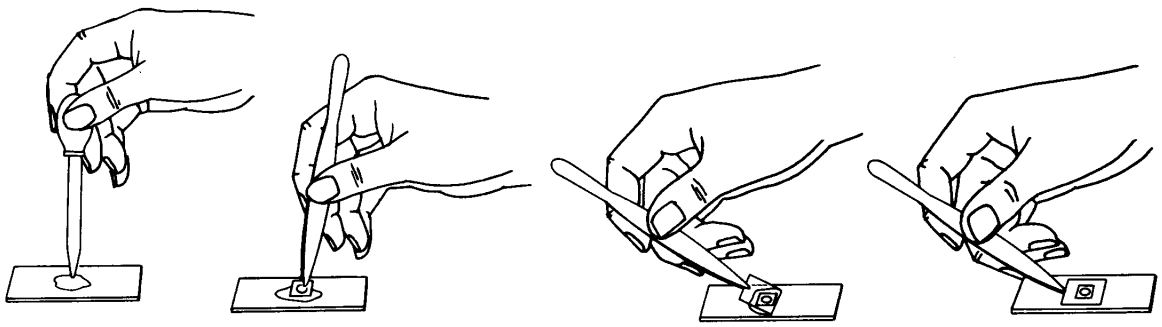
1. Cut out a lower case letter *e* from a newspaper or other printed page. Clean a microscope slide and prepare a "wet mount" of the letter, using the procedure described in Fig. 1-2. Put the scanning (4 ×) or low-power (10 ×) objective in position and then place the slide on the stage in its normal viewing position.

2. Clean the oculars and objectives using lens paper.

3. Turn on the illuminator and open the diaphragm fully. If there is a condenser, position it as high as it will go, so that the top lens of the condenser unit is level with the stage aperture.

4. Center the specimen over the stage aperture.

5. Position the scanning objective (4 ×) as close to the slide as possible and then, while looking through the oculars, use the coarse adjustment knob to back off slowly until the specimen comes into focus.



A. Add a drop of water to a slide.

B. Place the specimen in the water.

C. Place the edge of a coverslip on the slide so that it touches the edge of the water.

D. Slowly lower the coverslip to prevent forming and trapping air bubbles.

FIG. 1-2
Preparation of a wet mount slide.

6. Using the diaphragm (and/or adjustment of the transformer voltage), readjust the light intensity as necessary and again center the specimen by moving the slide.

7. Switch from the scanning lens to the low-power objective (10 ×). Make certain the objective "clicks" into position. If the specimen stays in focus, your microscope is **parfocal.** You can sharpen the focus by small adjustments of the fine adjustment knob.

If your microscope is not in focus after changing objectives, you may have to use the coarse adjustment knob followed by the fine adjustment knob. But remember, do not do this with the high-power or oil immersion objectives in position. Ask your instructor for help if you have difficulty focusing your microscope.

Recenter the specimen, adjust the diaphragm, and adjust the position of the condenser to increase the contrast of the specimen.

8. Switch to the high-power objective (43 ×) and adjust the focus using the fine adjustment knob.

These are the procedures usually used when examining a wet mount or a commercially prepared microscope slide. Always make your preparation using clean microscope slides. Always proceed from the lowest-power to the highest-power objectives, making minor corrections in focus and light as necessary. Learn to "fine tune" your microscope.

2. The Microscopic Image

The image you view in the microscope is affected by several factors: the orientation of the image, total magnification, the size and brightness of the field of view, the plane of focus, the depth of focus, and the contrast of the materials being examined.

a. Orientation of the Image

Hold the slide you made of the letter *e* so that the letter is in a normal reading position. Then, place it on the stage in the same position and examine it with the low-power objective. What difference is there, if any, in the way the image is oriented when viewed through the oculars as compared to looking at it directly with your eyes?

While looking through the microscope, attempt to make the image move to the right. In which direction did you have to move the slide?

Try to move the image up (away from you). Which way did you have to move the slide?

In what direction do you have to move the letter to make the image move right then up?

There will be times when you will want to show someone something of interest in the **field of view.** One way to do this is to describe its approximate location by referring to the field of view as a clock. Thus, you could tell them to "look at three o'clock," or "look just off center toward nine o'clock," and so

forth. Alternately, some microscopes have what appears to be a thin black line cutting across the field. This is a **pointer** that has been added to the ocular of your microscope so you can point out something by moving the object under observation to the end of the pointer.

b. Brightness of the Field of View and Working Distance

Examine your slide starting with the lowest-power objective and progressing to the highest-power objective. Describe any changes in the brightness of the field when you change objectives.

In Fig. 1-3 shade in the appropriate circles to correspond with any change in brightness you observed. When the object on your slide is in focus for each objective, the **working distance** between the slide and the objective lens decreases as the objective magnification increases. Of what value is such information to you?

If you know the diameter of the field for each magnification, you can use this information to estimate the size of object you are examining. To determine the diameter of the field, place a transparent millimeter rule on the stage, focus on the rule, and measure the diameter of the field for the scanning and low-power objectives. It will be very difficult to measure the high-power and oil immersion fields, but you can get a good approximation using the following formula, where

D_L = diameter of the field at a *lower* magnification
D_H = diameter of the field at the *higher* magnification
X_L = magnification of the *lower* power objective lens
X_H = magnification of the *higher* power objective lens

Thus

$$\frac{D_H}{D_L} = \frac{X_L}{X_H} \quad \text{or} \quad D_H = \frac{D_L \times X_L}{X_H}$$

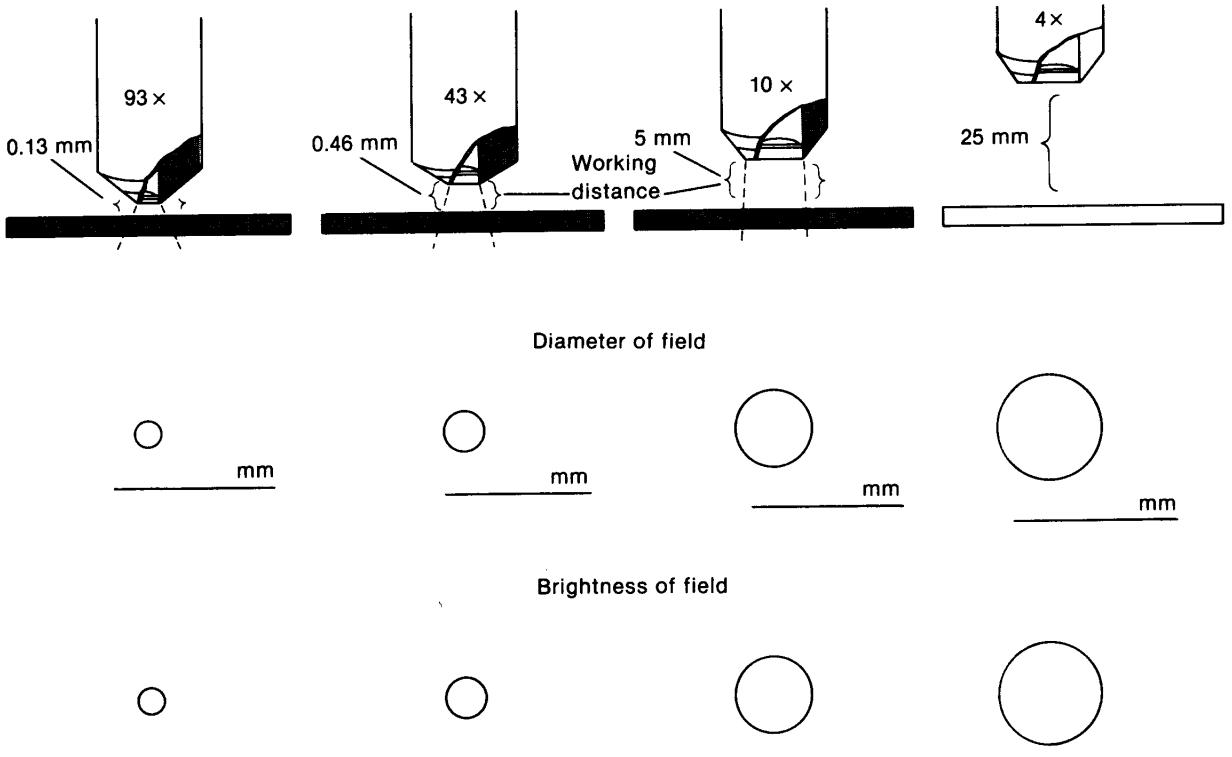


FIG. 1-3
Relationships between working distance, diameter, and brightness of field.

Insert the appropriate values in the formula and determine the diameters of the high power and oil immersion objective fields of your microscope. Record your data in Fig. 1-3.

c. Depth of Focus

Just like the human eye, the lenses of your microscope provide a limited **depth of focus.** This means that only part of the object will be in sharp focus, while areas above and below that part will be slightly out of focus or not in focus at all.

To become familiar with the concept of depth of focus, take your metric rule and, pointing it lengthwise away from you, hold it about 30 cm (12 inches) in front of you and about 7.5 cm (3 inches) below your eyes. Looking down the length of the rule, focus your eyes on the 10-cm mark. When this mark is sharply in focus, what numbers above and below the 10-cm mark are also in focus? What then is the depth of focus for your eyes?

_____

In practice, you will find that as magnification *increases,* the depth of focus *decreases.* You will have to learn to constantly use the fine adjustment knob when the higher power objective is in position. This will help you to determine something about the three-dimensional shapes of the objects under observation.

To visualize three-dimensional form and the concept of depth of focus, place a small strand of your hair and a white and a yellow thread across each other on a microscope slide. Add a drop of water and a coverslip. Using the scanning objective (4 ×), focus where the strands of hair intersect and determine the depth of focus at this magnification.

Change to the low-power objective (10 ×). Describe any changes in the depth of focus.

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Switch to the high-power objective (43 ×) and describe any changes in the depth of focus.

_____

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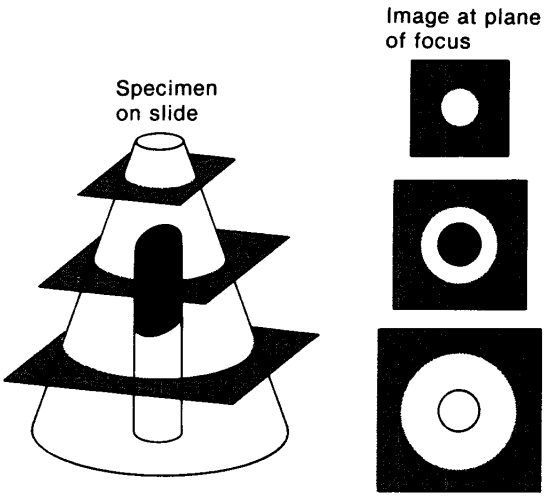

FIG. 1-4
Determining three-dimensional image through "optical" sectioning.

At this higher magnification, it is difficult but not impossible to determine three-dimensional form. You can do this by building a series of **optical sections** in your mind as you focus through the specimen. Fig. 1-4 demonstrates how this is done.

Try to determine the three-dimensional structure of your preparation at high power by making and visualizing a series of optical sections. Begin by focusing on the surface of the top thread and working through to the lower surface of the bottom thread or hair.

d. Contrast

Even with sufficient magnification and resolution, you can only visualize an object under a microscope if there is sufficient contrast between the object and its surroundings or between the various parts of the object.

Cells or subcellular structures may contain naturally occurring pigments (e.g., chlorophyll in chloroplasts, hemoglobin in red blood cells) that provide contrast and make these structures visible. Frequently, however, cells and parts of cells are highly translucent. One way to improve contrast is to use dyes or stains that bind to or are taken up by various subcellular structures and thus absorb enough light to provide the necessary contrast. In addition to staining, or in combination with it, you can improve image contrast by regulating the opening of the diaphragm. This deflects the light rays from edges of the diaphragm and causes them to enter the specimen at an angle. Such scattering of light makes the specimen look darker, since some of the light takes longer to reach the eyes.

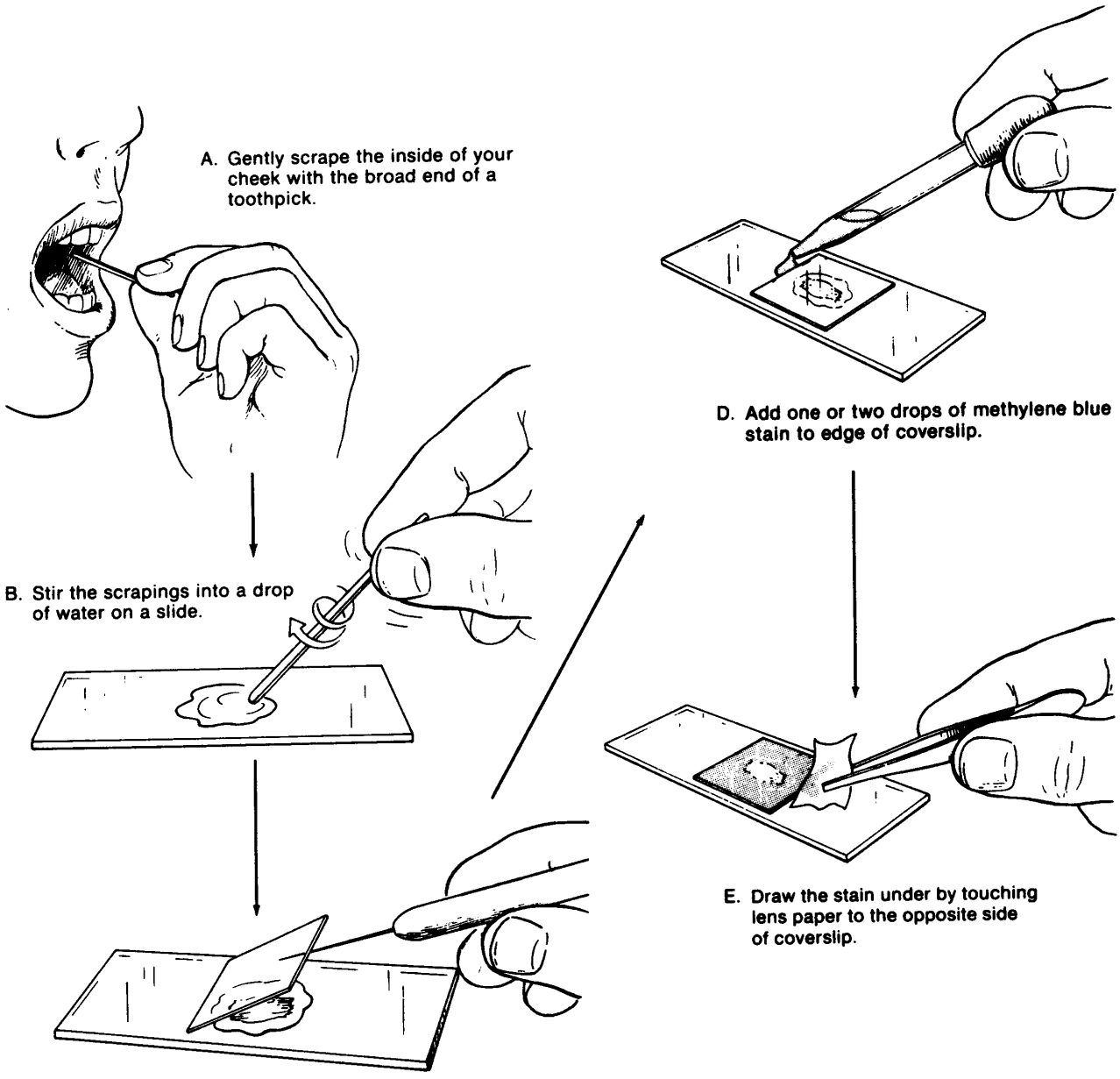

FIG. 1-5
Staining cells to improve image contrast.

Following the instructions in Fig. 1-5A–C, examine cells obtained from the inner epithelial lining of your cheek. Try to determine something of their structure by adjusting the diaphragm and the condenser. Add a drop of methylene blue stain to the edge of the coverslip and draw it under as shown in Figure 1-5D, E. Describe any changes in contrast, or visibility of the structures, in the cell.

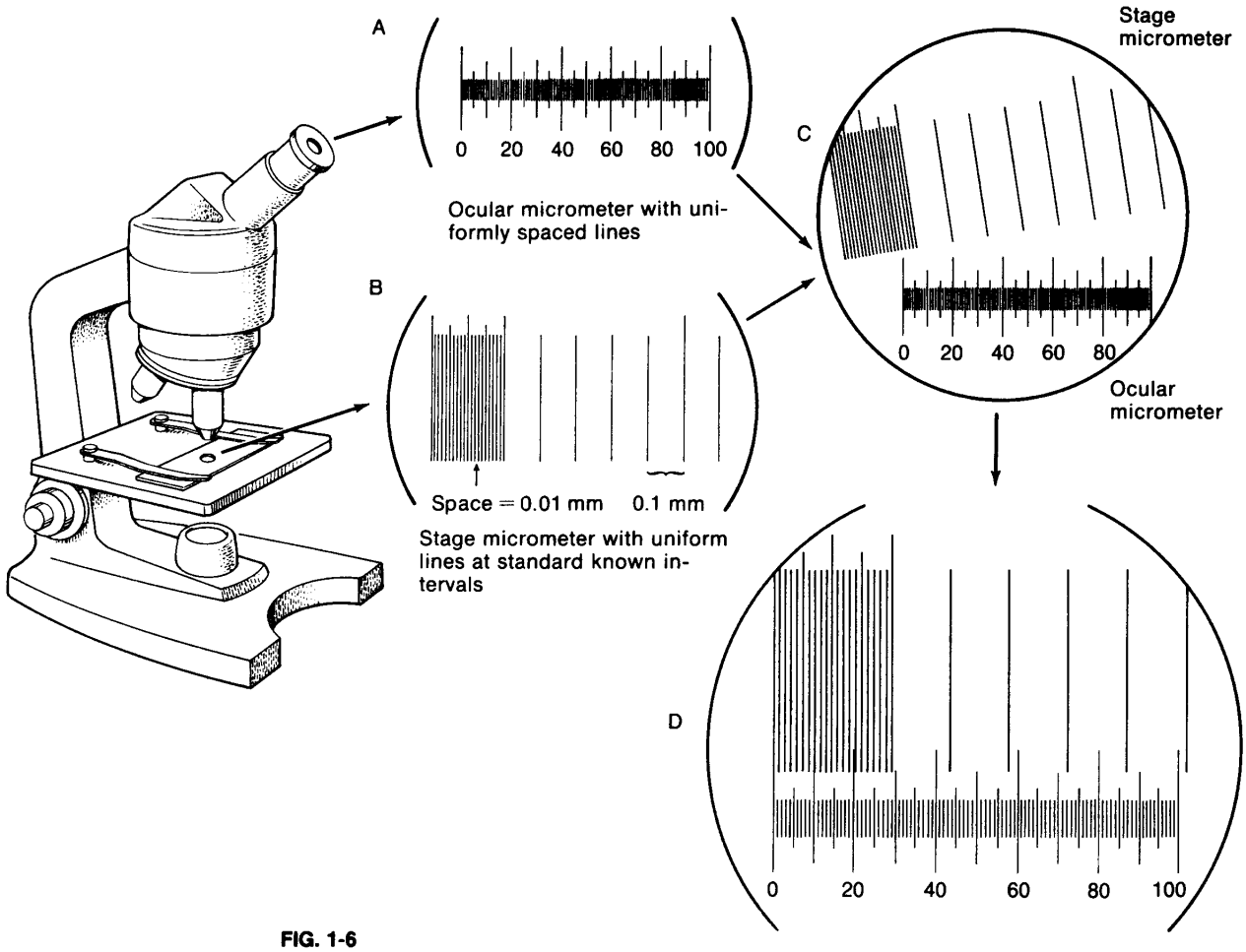

FIG. 1-6
Using an ocular micrometer to determine the size of microscopic objects.

e. Measurement of Microscopic Specimens

Recall that you earlier determined the diameter of the field of view of the various objective lenses on your microscope and, comparing the size of the specimen with the diameter, you obtained a rough estimate of the size of the object.

A more precise method involves using an **ocular micrometer,** a small glass disc on which uniformly spaced lines of *unknown distance* are etched. The ocular micrometer is inserted into the ocular of the microscope and then calibrated against a **stage micrometer,** which has uniformly spaced lines of *known* distances (Fig. 1-6). To calibrate the ocular micrometer use the following procedure:

1. If you were to observe the stage micrometer without the ocular micrometer in place, it would appear as shown in Fig. 1-6B. If you were to observe the stage micrometer with the ocular micrometer in place, it would appear as shown in Fig. 1-6C.

2. Turn the ocular in the body tube until the lines of the ocular micrometer are parallel with those of the stage micrometer. Match the lines at the left edges of the two micrometers by moving the stage micrometer (Fig. 1-6D).

3. Calculate the actual distance in micrometers (μm) between the lines of the ocular micrometer by observing how many spaces of the stage micrometer are included within a given number of spaces on the ocular micrometer. Since the smallest space on the stage micrometer equals 0.01 millimeter (mm), you can calibrate the ocular micrometer using the following:

10 spaces on ocular micrometer
$= X$ spaces on stage micrometer.

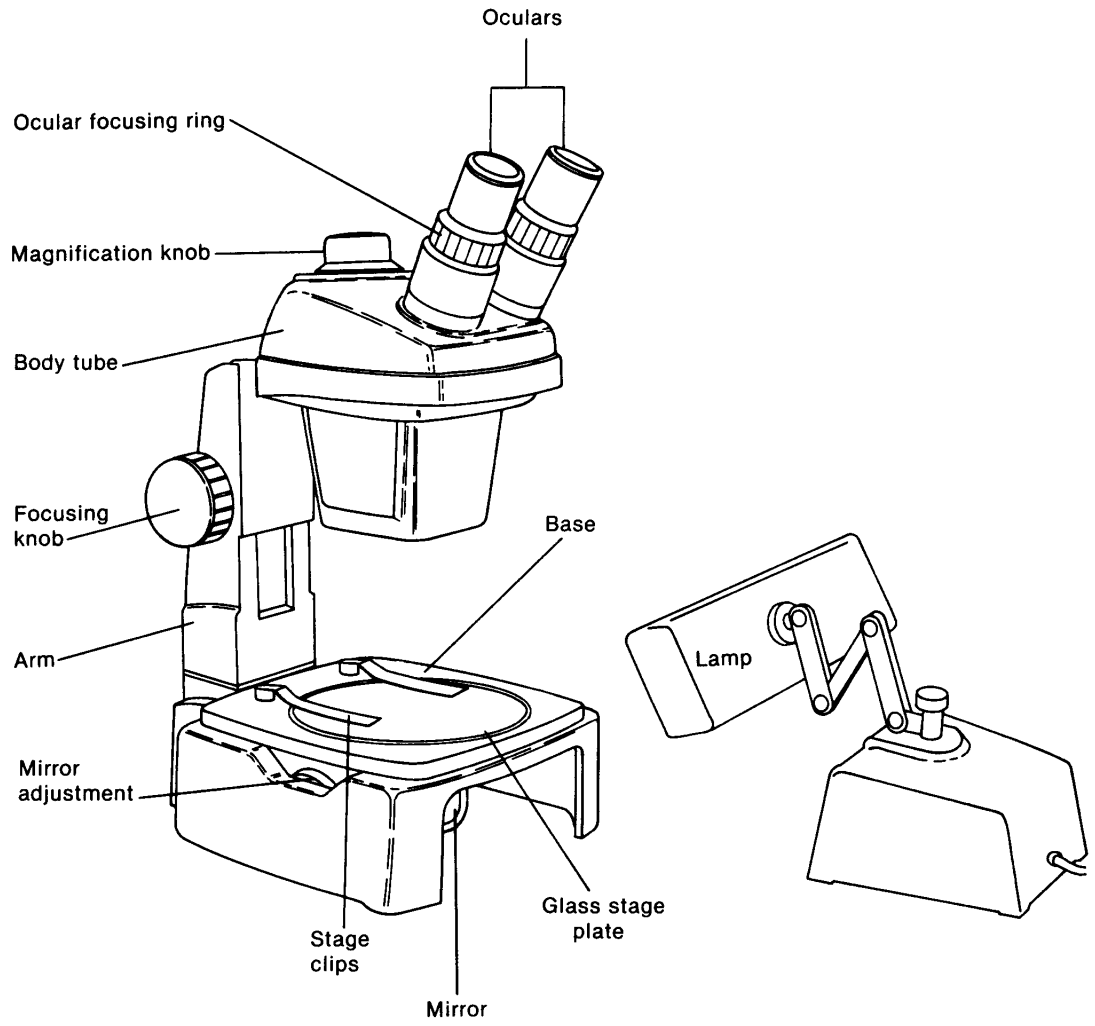

FIG. 1-7
Parts of a stereoscopic (dissecting) microscope.

Since the smallest space on a stage micrometer = 0.01 mm, then

10 spaces on the "ocular"
$= X$ spaces on the "stage" $\times$ 0.01 mm

and

1 space on the "ocular"

$$= \frac{X \text{ spaces on the "stage"} \times 0.01 \text{ mm}}{10}$$

Example: If 10 spaces on the "ocular" = 6 spaces on the "stage" then

$$1 \text{ ocular space} = \frac{6 \times 0.01 \text{ mm}}{10}$$

Of course, the numerical value obtained holds only for the specific objective–ocular lens combination used. Each time the objective or ocular lens is changed, the ocular micrometer will have to be recalibrated.

D. USE AND CARE OF THE STEREOSCOPIC (DISSECTING) MICROSCOPE

The stereoscopic dissecting microscope shown in Fig. 1-7 has two distinct advantages over the compound microscope: (1) it enables you to examine objects that are too large or too thick to be seen with the higher magnifications of the compound microscope and (2) it gives you a three-dimensional view of the specimen.

The stereoscopic microscope is often used when dissecting specimens. The light source may be reflected from an illuminator above the specimen or,

on some microscopes, transmitted through the specimen from a mirror below the stage. The choice of the light source depends upon whether the specimen is transparent or opaque.

Using your dissecting microscope, examine your fingers or some other opaque object. Adjust the oculars for interpupillary distance and focus as previously for the compound microscope (Part A-1). Change the magnification using the magnification knob on the top of the body tube. On other stereoscopic microscopes, the magnification is varied by switching ocular lenses, as with the compound microscope. How does the movement of the image compare to that of the compound microscope (Part C–2 of this exercise)?

How do you adjust the brightness of the field?

Examine the previously prepared slide of the crossed threads or hair. First use reflected light from the mirror, then use transmitted light from a lamp. Describe any advantage of one type of lighting over the other.

E. STUDY OF POND WATER

In your laboratory work, many observations made by the microscope will be on living organisms or on tissues or parts of organisms that you will want to keep alive. To allow them to dry out would greatly distort them, to say nothing of the effect death would have on a study of their movements. To observe living material prepare a wet mount of a drop of pond water as shown in Fig. 1-2.

Excess water under the coverslip can be soaked up by carefully placing a piece of paper toweling to the edge of the coverslip. However, if your preparation begins to dry out while under observation, add one drop of water at the edge of the coverslip.

Under low power and with reduced light, survey the drop of pond water. Identify as many of the organisms as you can. Carefully study their differences in structure and their method of movement. Figs. 1-8, 1-9, 1-10, and 1-11 should help you identify what you see.

Prepare additional wet mounts by taking samples from different parts of the jar of pond water. Do not be too hasty in discarding a slide because you don't find any microorganisms; a systematic survey of the preparation is often necessary to locate the organisms. Why do the organisms often accumulate at the edge of the coverslip?

To identify the smaller organisms, you may have to use the high-power objective. When your work is completed, clean and dry any slides and coverslips used. Wipe the lenses of the microscope with lens paper, clean the stage, and return the microscope to the cabinet.

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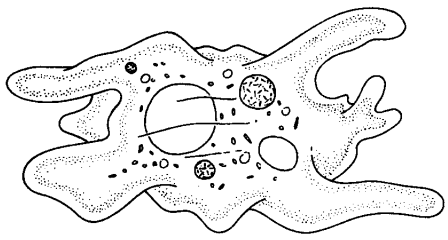
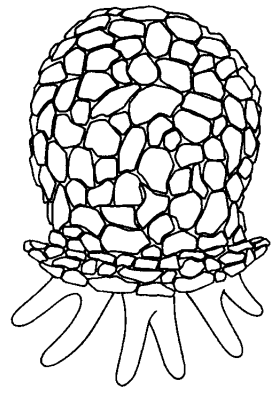
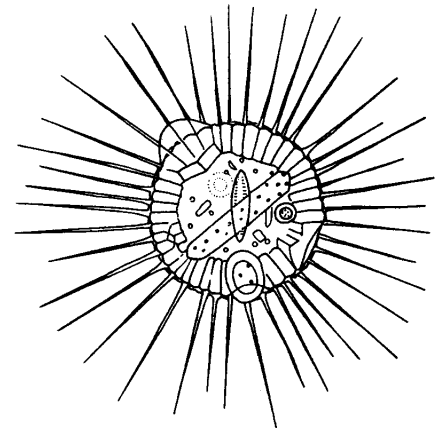


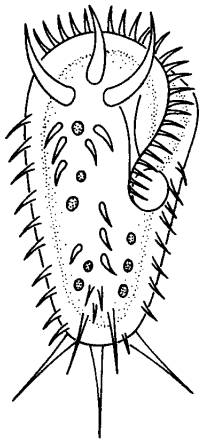
Amoeba



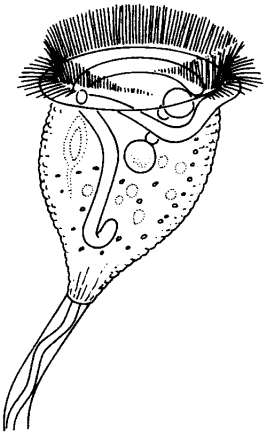
Difflugia



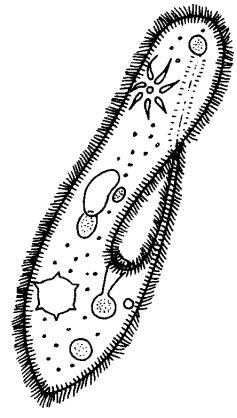
Actinosphaerium



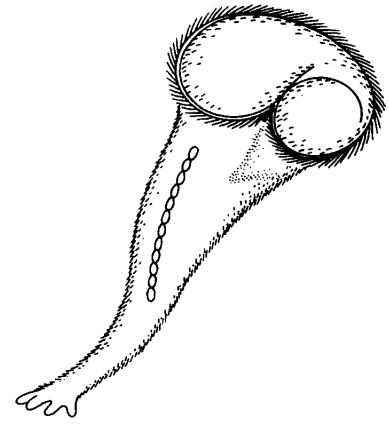
Stylonychia



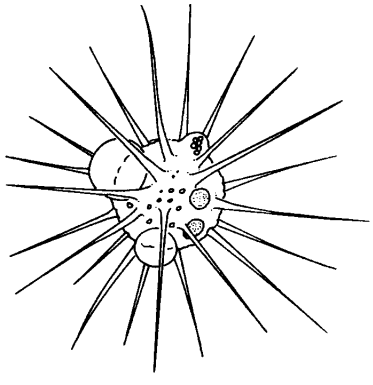
Vorticella



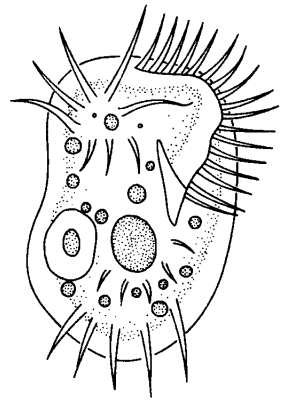
Paramecium



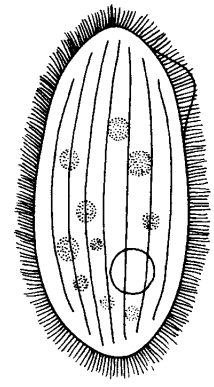
Stentor



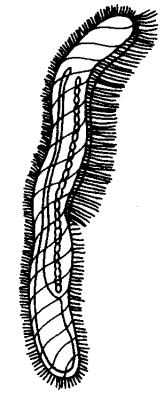
Actinophrys



Euplotes



Colpidium



Spirostomum

FIG. 1-8
Protozoans commonly found in pond water.

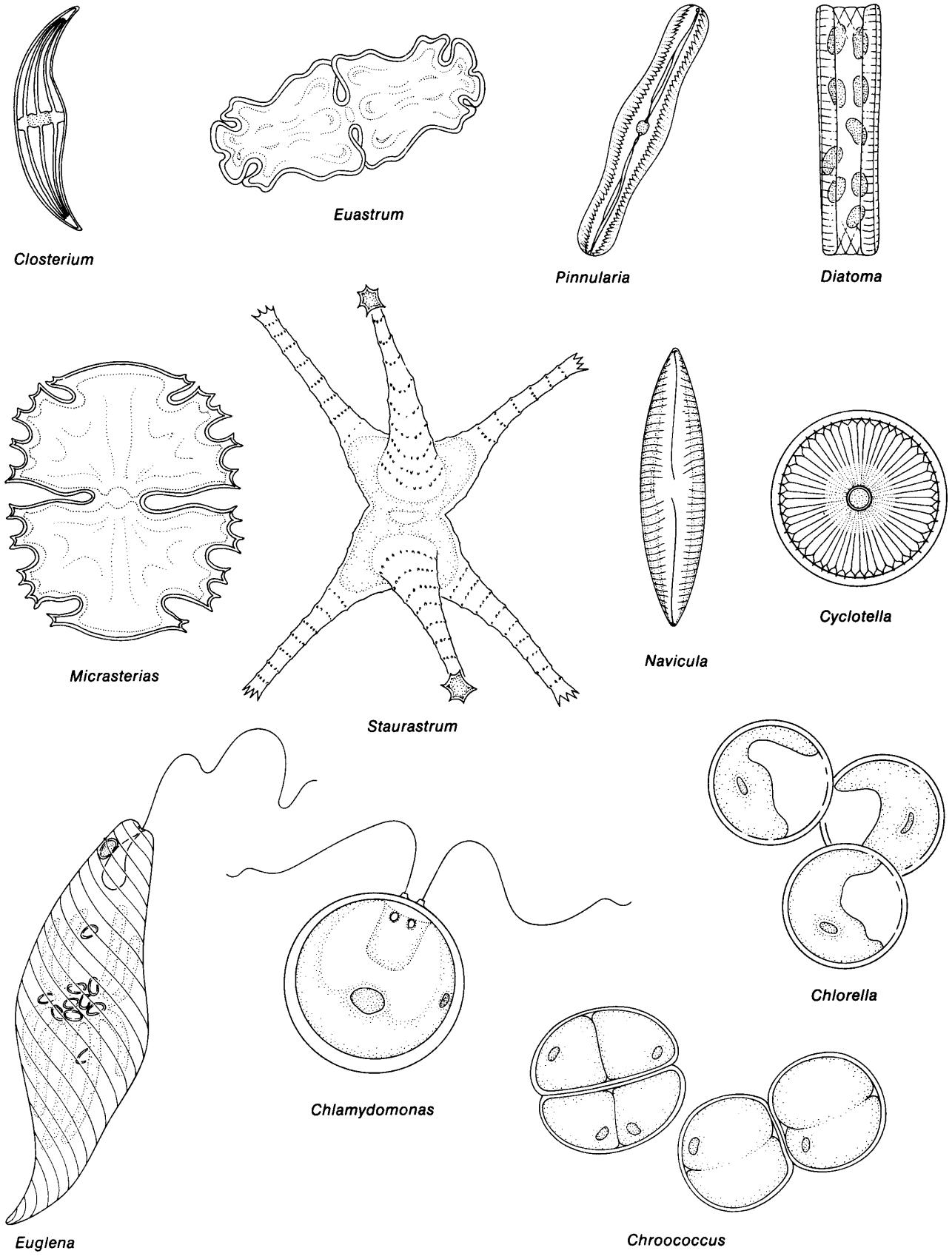


FIG. 1-9
Unicellular algae commonly found in pond water.

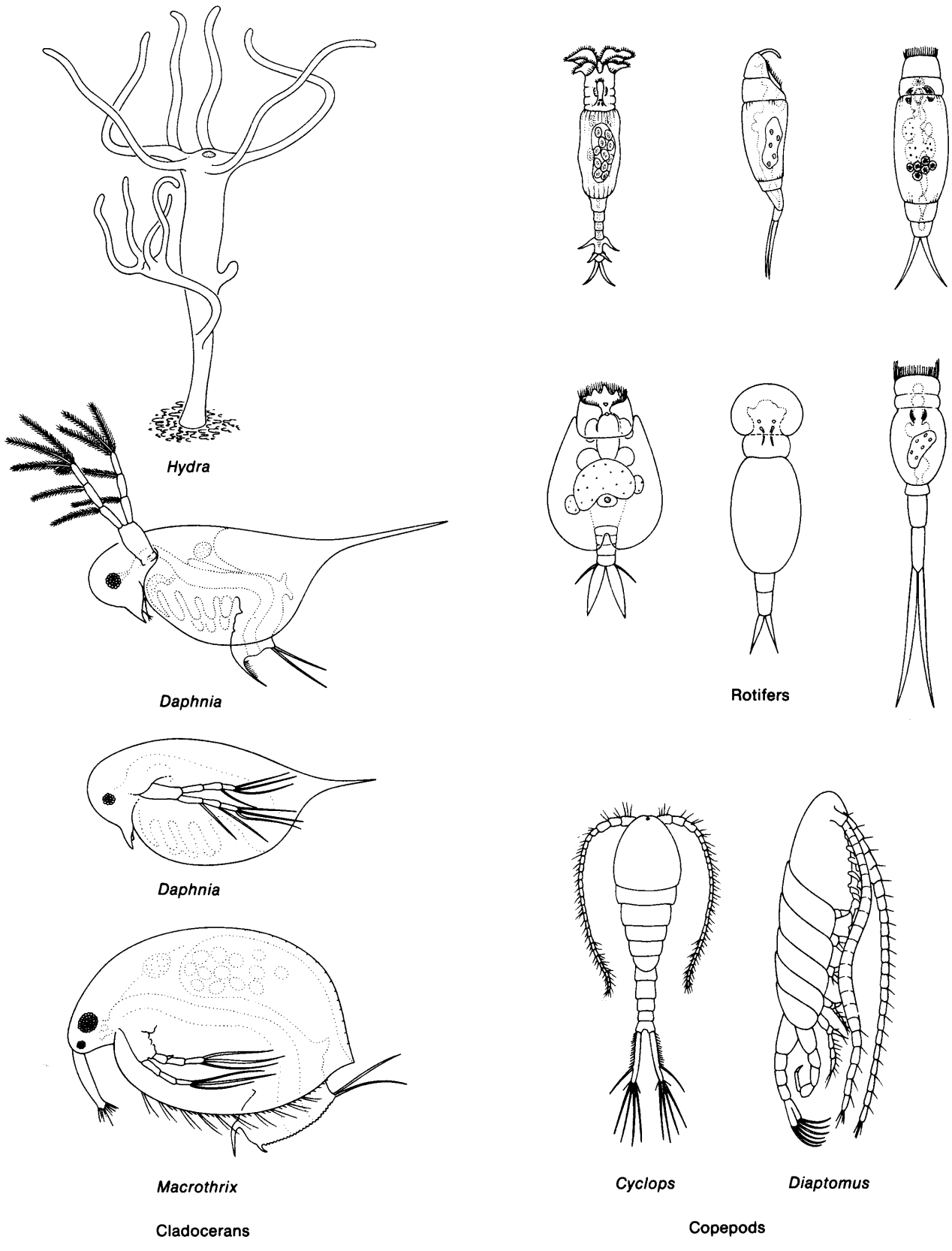


FIG. 1-10
Invertebrates commonly found in pond water.

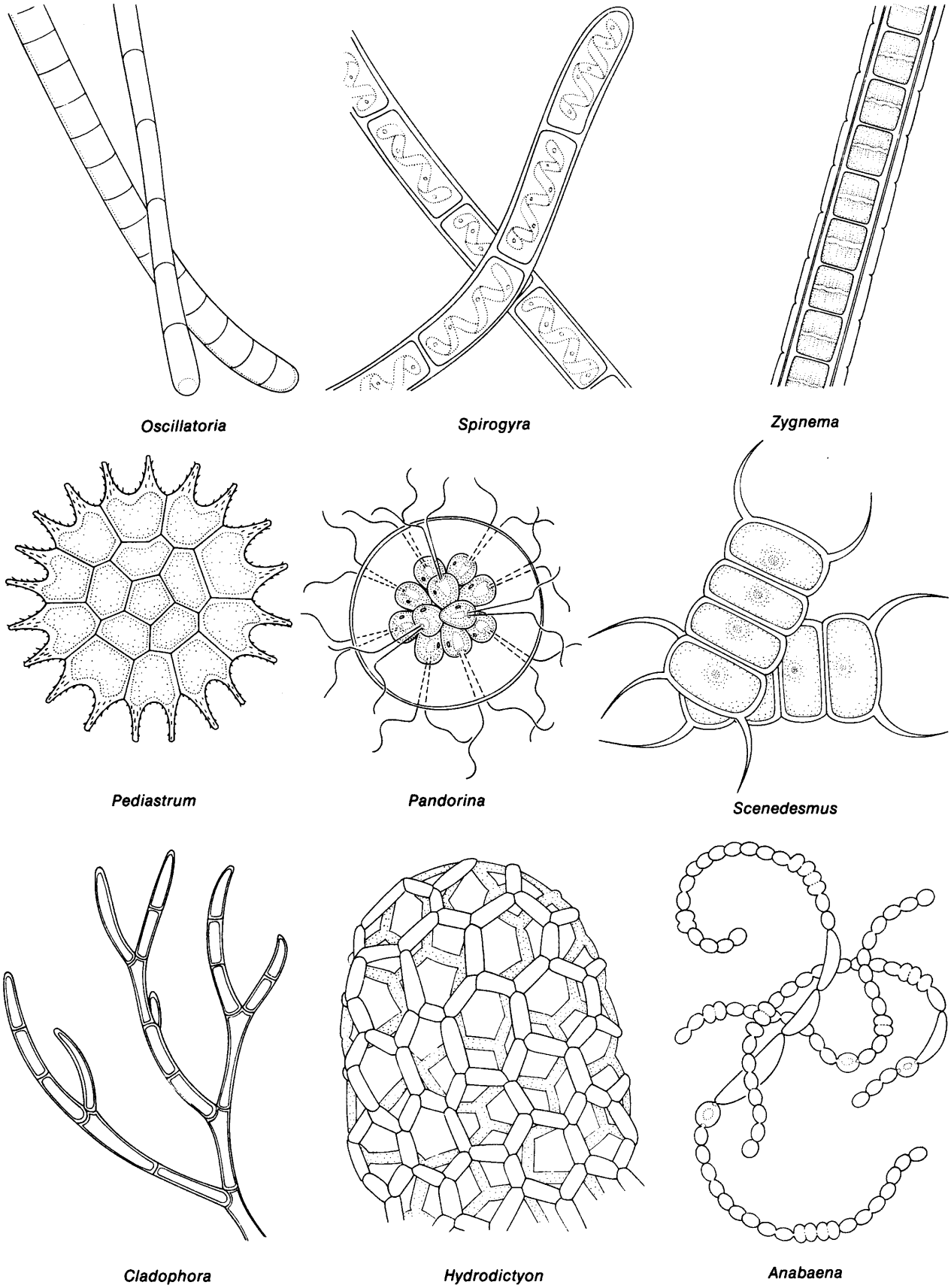


FIG. 1-11
Multicellular algae and cyanobacteria commonly found in pond water.

IX. Glossary of Selected Terms

ablation Extirpation, removal or otherwise damaging the eye in some manner to promote maturation.

Artemia Brine shrimp.

aseptic Sterile.

axenic Free from other living organisms.

bacteria One-celled organisms that can be seen only with a microscope. Compared to protozoans they are of less complex organization and are much smaller in size.

broodstock Larger animals that are sourced and expected to produce offspring in the maturation facility.

carboy Glass bottle.

cysts Eggs of *Artemia* that are in a dormant stage.

decapsulation Removal of the thick outer layer on *Artemia* cysts.

disinfection Reduction of bacterial numbers to "safe" or "acceptable" level.

exoskeleton Shell of the shrimp.

grooves There are numerous grooves on a shrimp. Some are used as an aid in identifying the species. Examples:

1) The "thumbnail" grooves on the last abdominal segment of tropical Atlantic brown shrimp distinguish them from similar white shrimp;

2) The length of the rostral groove is shorter in *P. monodon* than in *P. semisulcatus* and can be used as one way to distinguish the two similar species. Other distinguishing characteristics can be seen in Figure 18.

hemacytometer Device used for counting algae cells.

hemolymph Blood.

Hensen-Stemple pipet Pipet used to take an objective water sample.

hepatopancreas Digestive gland.

larvae Plural of larva and the stage in the development of a shrimp's life cycle between the egg and the juvenile.

maturation The act of maturing; in this case egg development, mating and then spawning.

media Plural of medium and in this case refers to saltwater and nutrients or food added to promote proper growth either of algae, larvae, etc.

molt For shrimp, shedding of the exoskeleton.

nauplii Plural of nauplius and the first of three major larval stages. It is a non-feeding stage and is the best stage to transport the animals until they reach the postlarval stage.

penaeid The family, superfamily and an infraorder of shrimp distinguished from the caridean shrimp by the shape of the second segment of the abdomen. The sides of the penaeid shrimp shell (known as the pleura) overlap each segment that is behind it. In the caridean shrimp the pleurum of the second segment overlaps both the first segment and the third, making the second segment look very large. There are 109 species of penaeid shrimp listed by the Food and Agriculture Organization of the United Nations (F.A.O.).

petasma Male shrimp reproductive structure.

prawn According to Dore and Frimodt (1987), different people use this name to mean and to apply to quite different species.

U.K.= larger than shrimp.
U.S. = restaurants use it to mean large shrimp and other places mean small shrimp or freshwater shrimp.
Norway = producers promote the northern shrimp *Pandalus* as a prawn, and the "Dublin Bay prawn" is even used to describe *Nephrops*, which is a langoustine or Norway lobster.

The Oxford Dictionary defines prawn as "larger than shrimp" whereas Webster's Dictionary describes it as "a small, edible crustacean of the shrimp family."

South Africa = larger animals = prawns; smaller animals = shrimp.

F.A.O. attempted to introduce a clear-cut distinction as early as 1967. At the World Conference on the biology and culture of shrimps and prawns held in Mexico City, it was agreed that the term "prawn" was to be reserved for freshwater creatures only, while their marine/brackish water relatives were to be called "shrimps." Unfortunately, despite all of their efforts the confusion continues. The only point on which everyone can surely agree is that the use of "prawn" in the English language is confusing and unclear, and should be avoided.

protozoea New world term for zoea or the second major larval stage of penaeid shrimp.

raceway A small pond or tank which is usually rectangular with a center divider or circular tank with a water flow that "races" around the tank.

rostrum The pointed prow that extends from the head of most shrimp.

setae Hair-like structures that appear to branch off of appendages or legs.

sourcing Obtaining animals to be used for broodstock.

spore A small cell that can develop into a new individual.

spp. or sp. Plural and singular abbreviations for species, respectively.

sterilization Total inactivation of all microbial life.

thelycum Female shrimp reproductive structure.

X. Acknowledgments

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