# 4. Post-spawning procedures

The post-spawning process includes the following components:

- larval-rearing unit preparation;
- larval-rearing management;
- health management;
- larval nutrition and feed management;
- important larval diseases;
- general assessment of larval condition;
- quality testing/selection of PL for stocking;
- PL harvest and transportation;
- nursery rearing;
- timing of PL stocking;
- use of multiple species in shrimp hatcheries;
- documentation and record keeping; and
- research and development and extension requirements.

## 4.1 LARVAL-REARING UNIT PREPARATION

In many cases, where hatcheries and farms form distinct economic units, larval quality is often sacrificed for economy or profit. However, in reality the most economic strategy is to produce PL that will grow quickly, are free from disease and that will give a high survival and production rate in the grow-out facilities. In order to achieve this, all areas involved in larval rearing must be designed for optimal efficiency, cleanliness and production of the best quality, high-health PL possible.

Entrance to the larval-rearing area(s) should be restricted to the personnel that work in these areas. Sanitary mats or footbaths containing a disinfectant solution (e.g. calcium or sodium hypochlorite solution, >50 ppm active ingredient) must be placed at the entrance of each room of the hatchery. The disinfectant solution must be replaced as necessary. At each entrance to the larval-rearing room(s), container(s) with povidone iodine (20 ppm) and/or 70 percent alcohol should be available, and all personnel must wash their hands in the disinfection solution(s) on entry to and exit from the rooms.

Each room should have a full complement of materials for routine operation (filters, meshes, buckets etc.). A tank of approximately 500–600 litres containing disinfectant (hypochlorite solution, 20 ppm active ingredient) should be provided to disinfect hoses, buckets etc. Common-use equipment can be placed in this disinfecting tank at the end of every day and rinsed before re-use the following day. The disinfectant in this tank should be replaced daily or as required.

Additionally, beakers, nets etc. used for each tank should be maintained in a bucket filled with sodium hypochlorite solution (20 ppm active ingredient) and dedicated to that one tank to prevent cross-contamination between tanks within the same unit.

The infrastructure for larval culture consists principally of one or more units of larval and PL rearing tanks that are either used from nauplius to PL harvest or sometimes in two phases. The design of the larval-rearing tanks should support effective cleaning of waste and appropriate water exchange. The first phase comprises tanks for larvae from nauplius up to PL4-5, which should preferably have conical, “U” or “V” shaped bottoms with a perforated air pipe or airstones situated at the base of the tank. The second phase (if utilized) comprises larger, flat-bottomed tanks or raceways for PL or nursery culture and stocked at lower density with PL4-5 until harvest (typically at PL15). Supporting infrastructure (discussed in more detail in Section 2) includes a

water storage, treatment, heating and distribution system; an aeration system; live feed production facilities for algae and *Artemia* (and others); laboratories for health checks, bacteriology and feed preparation; offices and an area for packing and shipping PL.

Protocols for tank disinfection and preparation for stocking are given in Section 2.2.3. These procedures should be followed to minimize the risks of transferring disease from one cycle to the next or from one tank (or unit) to the others.

## 4.2 LARVAL REARING/HEALTH MANAGEMENT

There are many factors involved in managing larval rearing and health in the hatchery. Tight control must be maintained on all of these factors throughout the larval-rearing cycle if good numbers of high quality PL are to be produced. Some of the more common factors affecting larval health during the larval culture cycle (assuming that high quality nauplii have been stocked according to the methods outlined in Sections 1.12, 1.13 and 1.14) are shown in Table 15.

### 4.2.1 Stocking rate

To optimize the water quality and reduce stress levels for the growing larvae, it is important to stock the correct number of larvae and exchange water to maintain optimal water quality conditions throughout the larval-rearing phases.

Before stocking the nauplii into the larval-rearing tanks, the high quality, disinfected and washed nauplii should be counted by taking at least three small samples from the nauplius holding tanks and calculating the average. They should then be acclimated to the larval-rearing tank water conditions by flow-through of water from the larval-rearing tank until temperature and salinity levels are equal. The nauplii should then be stocked into the larval-rearing tanks. Stocking density should be between 75 and 120 nauplii per litre (75 000–120 000/tonne), assuming a full larval–rearing tank (even though it will only be 50 percent full at stocking).

Ideally nauplii from individual spawners should be reared separately in individual larval-rearing tanks to avoid cross-contamination and maintain traceability. At a

TABLE 15

**Some factors affecting shrimp larval health and possible control measures**

| Factor | Effects | Control measures | Standard |
|---|---|---|---|
| Excessive stocking density | • Stress<br>• Cannibalism<br>• Poor water quality | Reduce stocking density | 750–150 nauplii/litre |
| Poor water quality:<br>(A) sea water<br>(B) tank water | • Mortalities<br>• Late moulting<br>• Deformities | (A) Improve water quality by proper filtration, chlorination &/or sterilization<br>(B) Increase water exchange | • Filter < 5 µm<br>• Activated carbon Chlorination (>10 ppm) followed by neutralization<br>• UV or ozone<br>• 20–100% water exchange per day |
| High bacterial loading | • Mortalities<br>• Fouling<br>• Deformities | • Improve tank & water disinfection protocols<br>• Disinfect nauplii & live feeds used<br>• Use probiotics | Zero green Vibrio colonies and low levels of yellow vibrios on TCBS agar plates |
| Long stocking period | Increased infection rates of later stocked larvae | Limit number of days to stock hatchery | 3–4 d/ unit/hatchery |
| Poor feed (quality &/or frequency) | • Cannibalism<br>• Malnutrition<br>• Epibiont fouling<br>• Poor water quality | • Appropriate feeding programme<br>• Frequent checks on feed consumption & water quality | Feed every 2–4 h to satiation with high quality feeds |
| Poor quality and/or quantity of algae | • Mortality in zoeal stages<br>• Fouling of larvae | Routine counts & quality checks | Chaetoceros or Thalassiosira at 80 000– 130 000 cells/ml |
| Infected Artemia nauplii | Source of bacteria leading to mortality | • Decapsulation of cysts<br>• Disinfection of Artemia nauplii | Hydrogen peroxide or hypochlorite at 20 ppm active ingredient |

production rate of 400 000–500 000 nauplii/female and a stocking density of 75–120 nauplii/litre, this would entail conducting larval rearing in individual larval-rearing tanks with a capacity of four to six tonnes.

Unfortunately the current setup in most Indian hatcheries is not suitable for rearing larvae spawned from single spawners, as the tanks are too large (generally >10 tonnes each). If individual spawning and rearing is not possible, mixing of two or more batches of only WSSV-negative (tested by PCR) nauplii from negative spawners is recommended.

### 4.2.2 Water exchange protocols

Indian hatcheries commonly have issues with water quality due to inadequate checking, control and treatment of inlet water and inappropriate management practices such as inadequate water exchange. Daily water exchange during larval rearing varies depending on the stocking density, the larval stage, the feeding regime and the water quality conditions in the larval-rearing tanks. In general the quantity of water exchanged should be sufficient to maintain the critical water quality parameters. The optimal levels for these parameters are shown in Table 16. The most important parameters that must be controlled are ammonia, nitrite, bacterial loading and quantity of suspended or settled wastes.

The quality of the new water (treated as detailed in Section 2.3) used for exchange should always be better than the quality of the water in the tanks in terms of bacterial loading and toxic metabolite (ammonia and nitrite) concentrations. However, it should be similar in terms of temperature, salinity and pH to avoid stressing the larvae with an abrupt change in these parameters. Whenever new water is added, it should first be treated with 5–10 ppm EDTA for chelation of heavy metals and bacterial inhibition, 0.05 ppm treflan to kill fungi, and (if desired) probiotics at 1–5 ppm to populate the rearing water with beneficial bacteria. A generalized scheme for water exchange (in a 10-tonne tank) is shown in Table 16.

Normally larval-rearing tanks should be filled to only 50 percent of their full capacity with clean, disinfected, filtered seawater at 30–35 ppt and 28–30 °C prior to stocking with nauplii. Then, during zoea stage, about 10 percent (of a full tank) of new water (including the volume of live algae used) should be gradually added per day until the tanks are full by the time mysis stage is reached. Water is not exchanged during the delicate 3–5 d zoeal stages (length of zoeal stage depends on temperature) since they are very delicate and the water quality should be good because the shrimp biomass is low and feeding comprises mostly live algae, which does not foul the water.

During the mysis stages 10–30 percent of the water is exchanged per day. This is accomplished by first draining water out of the tanks and then refilling the tanks gradually. When exchanging water, it can be either drained from the bottom drain of

TABLE 16
**Water volume/exchange rate and mesh strainer size during different stages of larval rearing (for a 10-tonne larval-rearing tank)**

| Larval stage | Water volume (tonnes) | Drain water (tonnes) | Water exchange rate (%/d) | Flow-through | Mesh size | |
|---|---|---|---|---|---|---|
| | | | | | (µm) | (mesh) |
| Nauplius | 5 | - | 0 | - | - | - |
| Zoea | 5–10 | - | add 1 tonne | - | - | - |
| Mysis | 10 | 7–9 | 10–30 | - | 300 | 85 |
| PL1-4 | 10 | 6–9 | 30–40 | - | 400 | 65 |
| PL5-8 | 10 | 5–6 | 40–50 | - | 500 | 50 |
| PL9-12 | 10 | 2–5 | 50–80 | If required | 700 | 35 |
| PL13-16 | 10 | 1–4 | 60–90 | If required | 1 000 | 25 |

the tank or siphoned out from the bottom. In either case mesh-net strainers will be required to prevent the removal of the larvae along with the water and wastes. The size of the mesh required will vary depending on the larval stage and should be changed according to the data given in Table 16.

Due to the possibility of stress on the delicate zoeal and mysis stages, some operators prefer not to change any water until the PL-1 stage is reached. In this case water can be added daily as for zoea and only exchanged after metamorphosis to PL.

During the early PL stages, water is exchanged in the same way but at greater exchange rates, aiming for 30–40 percent/d for PL1-4 and 40–50 percent/d for PL5-8. Later PL stages have greater requirements for water exchange, and rates should be increased to 50–80 percent/d for PL9-12 and 60–90 percent/d for PL13-16. If high levels of waste, ammonia, nitrite and/or bacteria are encountered, the water level in the tanks can be lowered and then new water allowed to flow through the tanks for some time, before refilling the tanks.

Typically in Indian hatcheries, less water than the recommended rates (as shown in Table 16) is used. Under these circumstances the major water quality problems encountered are the accumulation of unionized ($NH_3$) ammonia and nitrite ($NO_2$) above the 0.1 ppm critical concentrations, resulting in sublethal toxicity and allowing the proliferation of *Vibrio* sp. bacteria. To counteract this it is recommended that more water is exchanged (if available) and biological filters and/or ammonia and nitrite-consuming probiotic bacteria are used to control the levels of these toxic metabolites and repress the growth of pathogenic vibrios.

### 4.2.3 Siphoning of wastes

In addition to daily water exchanges, the bottom of the tanks will need to be siphoned from zoea 2-3 throughout larval rearing. Uneaten food and faeces may need to be siphoned from the bottom of the tanks periodically (although the use of a good probiotic will minimize this requirement). This should be done by turning off the air and allowing the larvae to come to the surface of the tank. If excess sediment/wastes can be seen (using a light) on the tank floor, siphon them into a net and then transfer the contents of the net into a bucket. Any larvae siphoned from the tank can then be returned to the tank.

### 4.2.4 Aeration

Uniform aeration in all parts of the tanks should be provided through use of a perforated air pipe or air diffuser stones (1 stone/$ft^2$) situated at the base of the tank to help promote thorough oxygenation and turn-over of the water in the tank and to keep the larvae and the feed uniformly distributed in the tank.

### 4.2.5 Water quality monitoring

Water quality parameters of temperature, salinity, pH (optimum 7.8–8.2), ammonia (optimum <0.1 ppm $NH_3$), nitrite (optimum <0.1 ppm $NO_2$) and bacterial concentrations should be monitored daily (or every two days) in each tank and recorded on data sheets to ensure optimal conditions are maintained throughout the larval-rearing period. The results should be analysed and together with the results of the analysis of larval quality, used as a basis for determining the water exchange and disease treatment requirements for each tank. Water quality and health monitoring records must be kept at each larval-rearing tank, as well as in a central recording at the managers' office.

If any disease or water quality problems are noted, the water exchange rates mentioned above should be increased. Water temperature should be maintained at 28–30 °C day and night throughout the larval-rearing period (or increased to 32 °C for zoea), while salinity should be maintained at 30–35 ppt until acclimation for pond conditions from PL10-12 onwards occurs, if required.

Currently most Indian hatcheries have to shut down and dry out their operations periodically due to supposed chronic *Vibrio* infections, which cause massive mortality after a few continuous larval-rearing cycles. It is theorized that these vibrios are colonizing and forming resistant (even to scrubbing with detergents and formalin) microbial biofilms on the inner walls of the water pipes and larval-rearing tanks that support colonization with vibrios and their liberation into the water and subsequent infection of the larvae, often by the luminous *Vibrio harveyi*.

Recommendations for resolving this problem are as follows:

- routine monitoring (using bacteriology with TCBS agar) of *Vibrio* levels in the entire hatchery system to identify their source and hence facilitate eradication or suppression;
- appropriate clean-up, disinfection and dry-out procedures for facility, pipes and tanks;
- application of a steam gun between the culture cycles on concrete tanks to kill bacteria;
- proper protocols for filtration and disinfection of incoming seawater (see Section 2.3);
- effective disinfection of broodstock, eggs and nauplii prior to stocking (see Sections 3.1.3 and 3.6);
- rapid stocking of the entire hatchery (or at least each unit) within three to four days to prevent contamination of young larvae;
- use of separate equipment for each tank and routine disinfection of equipment, hands and feet when in production (see Sections 2.2 and 2.4);
- use of disposable plastic covers for larval-rearing tanks, at least until the PL stages;
- use of clean stocks of live algae;
- decapsulation and disinfection of *Artemia* nauplii prior to feeding;
- maintenance of optimal water quality through larval rearing by water exchange, siphoning, feed control and testing;
- reduced reliance on antibiotics whose overuse leads to drug-resistant strains of pathogenic bacteria; and
- application of effective probiotic bacteria throughout the larval-rearing cycle to out-compete and suppress pathogenic vibrios.

Following these protocols will not only help minimize disease problems due to *Vibrio* spp., but all other disease problems also.

### 4.2.6 Chemical/antibiotic use

Chemicals should be used only where absolutely necessary and then minimally and responsibly applied. A list of chemicals recommended as safe and suitable for use in shrimp hatcheries for specific reasons is shown in Annex 2.

In December 2005 the Government of India notified the Coastal Aquaculture Authority Rules, 2005, which lists the antibiotics and pharmacologically active substances banned for use in Indian aquaculture. This list is included as Annex III of this report.

MPEDA cites serious problems with the use of antibiotics including increased drug resistance of pathogenic bacteria, limits of the export potential for Indian shrimp and negative health consequences for consumers of contaminated shrimp. They therefore recommend that farmers attempt to replace the use of antibiotics with probiotics, comply with regulations governing the use of antibiotics, be conscious of and cautious with the health of consumers and gain awareness of the maximum permissible limits and withdrawal periods for antibiotics whose use is permitted in aquaculture.

The leaflet provided by MPEDA recommends that farmers and hatchery operators:

- check that the products they purchase are approved for use in aquaculture, clearly labeled and free from antibiotics;
- store chemicals correctly;
- only use chemicals approved for specific purposes;
- maintain detailed records of chemical use;
- verify that seed and feeds are tested to ensure freedom from antibiotics (and if not free, immediately inform MPEDA);
- be aware of recent developments and educated on the problems with antibiotic use;
- try to control the environment of the shrimp to prevent disease rather than treat their gross clinical signs;
- do not import or use chemicals that are not approved;
- try to use probiotics as replacements for antibiotics;
- maintain a withdrawal period before harvest if antibiotics are used; and
- obtain advice from MPEDA and other approved sources rather than from chemical salesmen.

In addition to this advice from MPEDA, it is recommended that a full compendium providing a list of all the banned drugs, a list of all permitted drugs, suitable withdrawal periods after use of antibiotics and guidelines and licensing requirement etc. be prepared through an expert consultation. Draft guidelines should be subjected to general discussion before submission to the Ministry of Agriculture, who should be charged with enacting and enforcing any laws generated.

Proper use of chemicals within the industry will also permit traceability, which will inevitably become a growing issue with exportation of cultured shrimp.

### 4.2.7 Use of probiotics to replace antibiotics

Hatchery managers should attempt not to use antibiotics during their operations. Antibiotics are problematic for many reasons including:

- they are often ineffective for major problems;
- they are expensive;
- some are dangerous to workers and consumers and/or illegal;
- they can create strains of bacteria resistant to antibiotics (even for humans);
- they can reduce the effectiveness of antibiotics to treat human sickness;
- they cause slow growth and low immunity in the larvae; and
- it is illegal to export shrimp containing certain antibiotic residues.

Currently many Indian hatcheries use a wide range of antibiotics but are gradually changing towards the use of probiotics. However, some problems are encountered with lack of knowledge on selecting good probiotics, inappropriate dose rates and lack of knowledge on proper probiotic procedures. Effective probiotics should be used prophylactically throughout the larval-rearing period. Probiotics confer a number of advantages over antibiotics for shrimp undergoing larval culture. These include:

- the use of probiotics is usually more effective and cheaper than using antibiotics;
- probiotics actually treat the cause of the problem rather than the symptoms as antibiotics do;
- there is no acquired immunity to probiotic bacteria, so they will continue working for a long time;
- probiotics are able to reduce levels of pathogenic bacteria through out-competing them for food and producing natural inhibitory compounds (i.e. bacteriocins) of their own;
- some probiotics utilize toxic metabolites (ammonia and nitrite) directly from the tank, thereby enhancing water quality;
- some probiotic formulae contain bacterial enzymes that are able to reduce levels of organic material including faeces, uneaten food and other particulate organics, leaving cleaner water and tank floors;

- some probiotics (i.e. *Lactobacillus* sp.) enter the guts of the shrimp where they assist digestion and serve as a source of nutrition and hence improve food assimilation and growth of the larvae and eliminate toxic metabolites at source; and
- some probiotics can compete for gut adhesion sites, limiting colonization with pathogens and perhaps activating the immune system .

Protocols for use of hatchery probiotics are as follows:

- Select a probiotic that has a good reputation and a high concentration (minimum $10^9$ CFU/g) of a large number of bacterial species, together with yeasts and enzymes for their effects in reducing organic matter accumulation in larval-rearing tanks.
- The probiotic is first weighed depending on tank size and dose rate, and then put into a bucket with seawater and aerated for 4–24 h. It is then filtered through a 100 µm (250 mesh) net. The solid material (bran carrier) should be discarded and the liquid filtrate added to the larval-rearing tanks daily. Typical dose rates are 1–3 ppm/d for zoea, 2–5 ppm/d for mysis and are 3–10 ppm/d for PL, according to the manufacturers' recommendations.
- Some probiotics can also be added to the larval feed or "bioencapsulated" in algae and/or *Artemia*/rotifers to help administer them into the larval shrimp body. This is an area of promising research and may have a good future in shrimp larval rearing.
- Do not use probiotics and antibiotics together since the antibiotics will also kill the beneficial bacteria in the probiotics.

### 4.2.8 Responsible use of antibiotics

Where use of antibiotics is unavoidable, the following guidelines should be considered:

- Only use antibiotics that are specifically recommended and approved for use in Indian aquaculture operations.
- Where the product will be consumed, strictly follow guidelines on withdrawal periods after use of antibiotics (generally for farmers).
- Do not use antibiotics prophylactically, but rather only where absolutely required to treat specific bacterial problems.
- Conduct laboratory-based (histopathological and bacteriological) analyses to ensure that the problem is caused by bacteria, since antibiotics are only able to treat diseases of bacterial origin.
- Before using antibiotics attempt to locate and remediate the root cause of the problem, since antibiotic use will only treat the symptoms of the problem and not the underlying cause, which is invariably associated with poor environmental control. If this is accomplished first, antibiotic use or (at least) repeat doses may not be required.
- Conduct sensitivity testing of the recommended antibiotics on the particular bacteria causing the problems. Antibiotic sensitivity discs are available cheaply from local companies and can be used on bacterial culture plates inoculated with the problematic bacteria to determine which antibiotics are capable of killing them. Only after this analysis is done should the selection of antibiotic be done. (This work may need to be done under trained supervision in a dedicated disease laboratory).
- Follow recommendations on dose rates and time of treatment for the particular antibiotic selected. Use of suboptimal doses for short periods may create problems with bacterial resistance, while overdoses result in bacterial contamination of the environment on discharge and may cause reduced growth rate, feed assimilation and/or survival in the shrimp themselves.

## 4.3 LARVAL NUTRITION AND FEED MANAGEMENT

Larval growth and survival and the water quality of the larval-rearing tanks depend to a large extent on the quality and quantity of food offered to the larvae. Optimization of feeding regimes helps maintain good water quality while promoting fast growth and high survival of the larvae and hence optimal production from the hatchery. Some of the major biosecurity issues and disease problems facing Indian shrimp hatcheries result from a lack of control over the nutrition and feeding regime used during larval rearing. Particular areas of concern include a lack of, or poor algal-culture facilities and protocols, poor feeding management, unchecked health and quality status of the feeds and poor or non-existent disinfection protocols for feeds.

All steps of feed preparation, especially of live feeds (algae, *Artemia* and others) are a critical control point (CCP), because feed can be contaminated through inappropriate management. All sources of live, fresh or frozen food should be considered from the point of view of pathogen risk. The source, treatment, storage and use of feed items should be reviewed and steps taken to ensure that they are safe and properly managed.

The feeding regime used in shrimp hatcheries should be based on the use of live/preserved algae for zoea and mysis. Dead *Artemia* nauplii (killed by freezing) may be used only for mysis stages (since mysis have difficulty catching live *Artemia* nauplii). However, some Indian hatcheries prefer not to feed the larvae with *Artemia* at all during the mysis stages. In this case, feeding directly with live newly hatched *Artemia* nauplii is started at the PL-1 stage (see subsequent sections for live feed procedures and dose rates).

However, these live diets require supplementation with artificial dry or liquid diets to achieve optimal production of larvae. Dose rates for artificial diets should be based on observations of the larval feeding habits and water quality, manufacturers' recommendations and experience. Care must be taken not to overfeed with artificial diets, as this may lead to water quality problems and fouling of the larvae. Algae dose rates are shown in the next section. *Artemia* dose rates are calculated based on the use of about 6–7 kg/million PL produced, while trying to maintain 3–5 *Artemia* nauplii/ml in the larval-rearing tanks.

It is difficult to detail exact feeding protocols for larval rearing. The feeding regimes should be based on the specific requirements of the various larval stages, backed by frequent and detailed examination of the feeding activity of the larvae in each tank. Indications are given in this section of significant points to bear in mind.

TABLE 17
**Guide to feeding regime for zoea and mysis stage larvae [1]**

| Day | Stage | Time | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0:00 | 2:00 | 4:00 | 6:00 | 8:00 | 10:00 | 12:00 | 14:00 | 16:00 | 18:00 | 20:00 | 22:00 |
| 1 | N/Z1 | | | | | A | | A | F1 | A | F1 | A | F1 |
| 2 | Z1 | A | F1 | A | F1 | A | F1 | A | F1 | A | F1 | A | F1 |
| 3 | Z1/Z2 | A | F1 | A | F1 | A | F1 | A | F1 | A | A | F1 | |
| 4 | Z2 | A | F1 | A | F1 | A | F1 | A | F1 | A | F1 | A | F1 |
| 5 | Z3 | A | F1 | A | F1 | A | F1 | A | F1 | A | F1 | A | F1 |
| 6 | M1 | A/D | F2 | A | F2/D | A | F2 | A/D | F2 | A | F2/D | A | F2 |
| 7 | M2 | A/D | F2 | A | F2/D | A | F2 | A/D | F2 | A | F2/D | A | F2 |
| 8 | M3 | A/D | F2 | A | F2/D | A | F2 | A/D | F2 | A | F2/D | A | F2 |
| 9 | M3/PL | A/D | F2 | A | F2/D | A | F2 | A/D | F2 | A | F2/D | A | F2 |

[1] Note: A = algae (live or preserved *Chaetoceros*, *Thalassiosira* or *Skeletonema* spp.); F1 = artificial feed (Fippak/Lansy/liquid feed/*Spirulina* etc) 10–80 µm; F2 = artificial feed (Fippak/Lansy/liquid feed/*Spirulina* etc) 50-150 µm; D = dead (well-frozen) *Artemia* nauplii (if required).

For zoea, microparticulate diets (i.e. microencapsulated Frippak CAR and Lansy Z-M and dried powdered *Spirulina*) or liquid emulsion diets (i.e. Epicore LHF or Cargill Liqualife) of 10–80 µm particle size are required. These should be fed alternately to the addition of live algae, up to six times per day (each for algae and formulated diets) over a 24-h period, to satiation. If the zoea maintain long trails of faeces and all stages of larvae are seen to have full guts at all times, then they are being fed enough. Care should be taken to ensure that overfeeding does not occur. This can be checked by examination of the amount of food in the water and the amount of faeces and uneaten food on the tank bottom.

For mysis, similar dry or liquid diets of 50–150 µm-sized particles should be fed in the same manner, as shown in Table 17.

For PL a variety of similar dry, liquid and crumbled flake diets of 200–300 µm-sized particles for PL1-8 and 300–500 µm from PL9-15 should be fed. This feeding is in the same manner, little and often, once every 4 h (six times/d) over each 24-h day period, alternating with six feedings per day of newly-hatched, live *Artemia* nauplii, as in Table 17.

For all of these artificial diets, sufficient aeration should be provided to maintain the dry or liquid feed particles in suspension at all times. This is so that they are always available to be eaten by the larvae and that they do not settle out onto the tank bottom where they are unavailable and may lead to deterioration in the tank water quality.

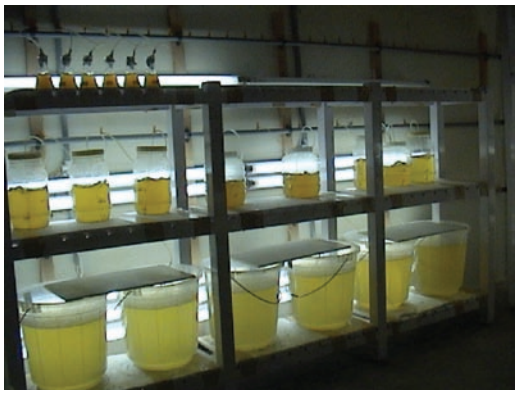
### 4.3.1 Use of live algae

Live (or preserved, if live are unavailable) diatom microalgae are the best food for early larval stage (zoea and mysis) shrimp. Not only are they self-suspending in the water column, they offer the perfect nutrition, contribute to enhancement of water quality (by absorbing ammonia, nitrite and carbon dioxide and producing oxygen), maintain shade in the water, produce natural and helpful bacteriocides and act as enrichment, increasing the nutritional value of the *Artemia* fed. Live algae should therefore always be offered to the early-stage larvae, as long as the algal culture can be kept clean.

However, one of the most common sources of pathogenic *Vibrio* spp., fungi and protozoa in larval rearing is the live algal cultures. Thus when culturing live algae, it is vital to start from a clean stock culture, maintain systems of hygiene and ensure that the algal-culture tanks, the equipment and the water used in them is clean and free from pathogens. General water disinfection procedures are provided in Section 2.3, but for algal culture, additional filtration of water and air (to 0.5–1 µm), and UV treatment of the water is required for all stages of production. Ozonation has also been used but any residual ozone can cause problems for algal growth and it is best avoided. Additionally all water, equipment and pure laboratory-grade fertilizing chemicals used in the algal-culture laboratory should be sterilized by autoclaving prior to use.

All algal-culture tanks must be washed and disinfected after each harvesting. Following disinfection of the tanks with calcium (sodium) hypochlorite solution (10 ppm active ingredient), they should be rinsed with clean, treated water and washed with 10 percent muriatic acid before being left to dry. The tanks should be plastic, fibreglass or epoxy-painted or plastic-lined concrete to aid cleaning operations and maintain clean algal cultures. If possible the tanks should be transparent or white in colour and the water depth should not exceed 1 m to increase light availability and algal growth.

Pure cultures of algae must be obtained and maintained using appropriate sanitary and microbiological procedures. The algal-culture laboratory should be well-lit with fluorescent tubes and air-conditioned to maintain temperatures between 18–24 °C. The algae are cultured here using extreme hygiene and sanitation to at least until 20-litre bag or carbuoy size. The algae are then passed outside the laboratory to cylinders or small tanks that are either well-lit artificially and/or in direct sunlight.



*Indoor algal culture with different phases of multiplication at a hatchery*

It is important to continuously monitor *Vibrio* concentrations (particularly the pathogenic green colonies) in the algal-culture system prior to feeding to maintain control over possible pathogens. Use of probiotic bacteria in the mass algal production systems has shown promise in maintaining clean algal cultures. It also doses the larval culture tanks with beneficial bacteria at the same time as adding the algae.

Due to the high risk of contamination from/to these areas, entry to the algal-culture and *Artemia*-culture rooms must be restricted to authorized personnel only, and staff from these areas should not be able to enter other production areas. A footbath containing a disinfecting solution (calcium (sodium) hypochlorite, >50 ppm active ingredient) should be placed at the entrance of each room. This solution must be replaced as often as necessary. As in other areas, container(s) of disinfectant solution (20 ppm of povidone iodine and/or 70 percent alcohol) should be placed at the doors and all staff should wash their hands on entering and leaving the room.

Single-celled algae such as *Chaetoceros*, *Thalassiosira*, *Tetraselmis*, *Isochrysis* and *Skeletonema* spp. are most commonly used. Pure cultures of all the algal species used should be maintained and cultured and subcultured on site, at all stages (from agar plates and tubes/bottles in the laboratory to massive on-growing outside). Alternatively pure starter cultures can be purchased from reputable algal-culture laboratories and be on-grown in the hatchery's massive tanks. Appropriate sanitary and microbiological procedures should be used to ensure the quality of the culture. The procedure of buying one lot of pure algal culture and continuously subculturing it throughout each larval culture cycle (commonly done with *Skeletonema* spp.), is not recommended, as it can easily lead to contamination of the algae and eventually, of the larvae themselves.

Once bloomed, the algae are examined using a microscope to determine cell concentration, growth phase, condition and presence of bacteria and protozoa. If free from contamination, at high density (>1 million cells/ml) and determined still to be in the highly nutritious log-growth phase, the algae is then pumped directly from the massive algal-culture tanks (through clean pipes) into the larval-rearing tank. Alternatively, with some species it can be filtered through a fine-mesh bag and the concentrate added to the larval-rearing tanks. Feeding with live (or preserved) microalgae is usually done two to six times per day, depending on the levels of algae remaining in the tanks after algal cell counts have been made.

The microalgae are usually offered to the larvae from the last naupliar stages (at 50 000–80 000 cells/ml), so that upon metamorphosis to the first feeding stage (zoea 1), the larvae will be able to begin feeding immediately. Concentrations are usually maintained at 80 000–130 000 cells/ml throughout the zoeal and mysis stages (peaking at Z3), and then decline to 50 000–60 000 cells/ml through the PL stages as the larvae become more carnivorous (as shown in Table 18). During PL or nursery culture, benthic algae are often used, as the PL will begin grazing algae from the walls of the tanks.

If concentrated preserved algal products are to be used, they should be purchased fresh and maintained in a refrigerator until required. They are then added up to six times per day in small quantities as per the manufacturer's recommendations, to maintain the desired algal cell densities as shown in table 18.

At least twice daily (and preferably six times per day), the number of algal cells in each tank should be counted (using a haemocytometer and a microscope) and

TABLE 18
**Algae maintenance regime for zoea, mysis and early PL stage [1]**

| Day | Stage | Time | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0:00 | 4:00 | 8:00 | 12:00 | 16:00 | 20:00 |
| 1 | N/Z1 | | | 50–80 | 50–80 | 80–100 | 80–100 |
| 2 | Z1 | 80–100 | 80–100 | 80–100 | 80–100 | 80–100 | 80–100 |
| 3 | Z1/Z2 | 80–100 | 80–100 | 80–100 | 80–100 | 80–100 | 80–100 |
| 4 | Z2 | 100–130 | 100–130 | 100–130 | 100–130 | 100–130 | 100–130 |
| 5 | Z3 | 100–130 | 100–130 | 100–130 | 100–130 | 100–130 | 100–130 |
| 6 | M1 | 100 | 100 | 100 | 100 | 100 | 100 |
| 7 | M2 | 100 | 100 | 100 | 100 | 100 | 100 |
| 8 | M3 | 80–100 | 80–100 | 80–100 | 80–100 | 80–100 | 80–100 |
| 9 | M3/PL | 80 | 80 | 80 | 80 | 80 | 80 |
| 10 | PL1 | 60–80 | 60–80 | 60–80 | 60–80 | 60–80 | 60–80 |
| 11 | PL2 | 60 | 60 | 60 | 60 | 60 | 60 |
| 12 | PL3 | 60 | 60 | 60 | 60 | 60 | 60 |

[1] Numbers are algal cells/ml to be maintained in larval-rearing tank.

compared to the desired range as shown in Table 18. Any deficiency in algal cell numbers should then be made up by adding appropriate volumes of algal cultures for which the cell density has already been established. Excess algal density should not be used since it may lead to fouling of the larvae. Regular monitoring of algal density in the larval-rearing tanks is crucial. This is especially the case with tanks open to the sunshine, where natural blooms will occur in the larval-rearing tanks with little need to add more algae beyond the first application, except where high water exchanges are conducted.

### 4.3.2 *Artemia* use

*Artemia* nauplii are the most important food item available to the shrimp hatchery. They are the main food fed to larval shrimp from early mysis all the way through to harvest of PL10-15 or more. Typically 6–7 kg of *Artemia* cysts will be fed for each million PL produced in *Penaeus monodon* hatcheries. Feeding with *Artemia* is conducted four to six times per day, always attempting to maintain 3–5 *Artemia* nauplii/ml in the larval-rearing tank so that they are always available to be fed on.

Because *Artemia* cysts are normally expensive and a potentially serious source of contamination, a number of procedures (involving decapsulation, hatching and disinfection) must be followed to optimize cost-efficiency of use while minimizing the risks of contamination of the larvae. Although the cost of *Artemia* cysts varies depending largely upon hatch rate, the best quality (highest hatch rate) *Artemia* available should be purchased since it is of superior nutritional quality and leads to optimized resource use in the hatchery. Whichever cysts are purchased, measures should be taken to ensure that the *Artemia* do not pose a risk of disease introduction. Certification may be requested for freedom from TSV, WSSV and YHV viruses by PCR analysis for all batches of *Artemia* cysts purchased.

#### Artemia *decapsulation*

Although *Artemia* cysts may not carry major viral pathogens, they are certainly a significant source of bacterial, fungal and protozoan diseases. Therefore decapsulation (removal) of the cysts is recommended to increase the hatching rate and avoid contamination of the *Artemia* culture water and the larval-rearing tanks with viruses, bacteria, fungi, microsporidians, other parasites and organic matter that can lead to disease and mortality.

*Placing* Artemia *hatching tanks in a separate room increases biosecurity*

First open the *Artemia* cans and pour the cysts into a large plastic bucket. Add freshwater and hydrate with continuous aeration for 1 h. Then put the hydrated cysts into a 80–100 µm (250–320 mesh) net and wash under running clean freshwater (preferably) or seawater.

There are two methods for decapsulation – method 1 (traditional) and method 2 (longer, but cheaper).

**Method 1:** Place the hydrated cysts back in the bucket and add 40 g of sodium hydroxide (NaOH) crystals and 4 litres of sodium hypochlorite liquid (chilled to 4 °C) and 4 litres of seawater (also chilled to 4 °C)/kg of cysts. Stir continuously for 5–8 min until the eggs begin to turn orange. Maintain the temperature at 18–25 °C through the addition of ice.

**Method 2:** Place the hydrated cysts back into the bucket and add about 7 litres of seawater and ice at a temperature of 20 °C. Add calcium oxide (CaO) at the rate of 125 g/kg of cysts. Stir until mixed. Then add calcium hypochlorite at the rate of 275 g/kg of cysts. Stir until mixed. Stir for 5–8 min, maintaining the temperature at <40 °C through the addition of ice.

Then add some more ice to decrease the temperature to 30 °C, add calcium oxide and calcium hypochlorite at the same dosages as before and stir for another 5–8 min until the eggs begin to change from white to orange in colour. The whole procedure will now have taken 10–16 min.

***Stopping the reaction and washing the eggs***

Immediately after the eggs have turned orange, transfer them into a 80–100 µm (250–320 mesh) net, wash with running freshwater (preferably) or seawater and then place into another bucket containing a solution of sodium thiosulphate crystals at 100 g/kg of cysts in a little water and stir until mixed. This will neutralize the remaining chlorine and stop the reaction. The decapsulated eggs will then sink and any floating debris can be discarded. Then collect the decapsulated eggs, wash once again and they are then ready for hatching or preservation in salt.

***Preservation***

There are two methods for storage of the decapsulated eggs if they are not required immediately. They can either be preserved in a solution of super-saturated brine (highly concentrated seawater at about 300 ppt) or in dry refined salt at about 300 g NaCl/kg of eggs. The first technique keeps the eggs in good condition for about one week, during which time they should be used. The second technique can preserve the eggs for up to seven weeks. If using the second technique, drain off the water produced during the salting process and store the eggs in the shade at room temperature or in a fridge.

**Artemia *hatching***

Specific *Artemia* hatching techniques should be used to obtain the highest possible number of clean nauplii from each can of cysts hatched. These techniques are necessary to produce clean *Artemia* for feeding the larvae at the lowest possible cost.

Clean and disinfect the *Artemia* hatching tanks by washing with water, scrubbing with a chlorine-soaked brush or cloth, washing again and then filling with clean, disinfected seawater (>10 ppm chlorine for >12 h, then dechlorinated with sodium

thiosulphate at 1 ppm for every 1 ppm of chlorine remaining). To further improve the hatching rate, instead of full-strength seawater, 18–25 ppt seawater (made from seawater diluted with clean freshwater) should be used.

Probiotics antagonistic to vibrios may be used in the *Artemia* hatching tanks to ensure the *Artemia* nauplii are free from pathogens when fed to the larvae. Alternatively 30–60 ppm chloramine-T may be added to the hatching tank together with the cysts and the cysts hatched in this solution. The use of this product can result in *Artemia* nauplii virtually free from contamination after this procedure.

Add the decapsulated cysts to the conical *Artemia* hatching tank at <1 kg cysts/1 000 litres of water (1 can of 425–450 g/500 litre tank). Place one or more airstones in the tank and provide full and constant aeration. Switch on a light placed 30 cm above the centre of each tank and leave it on constantly (day and night). After about 20–24 h (when most of the cysts have hatched), or 12–18 h for umbrella stage, switch off the air, allow the nauplii and debris to settle and harvest the live nauplii from the tank into a 100 µm (250 mesh) net. Wash thoroughly under clean running freshwater (preferably) or seawater then disinfect.

**Artemia *nauplius disinfection***

Specific techniques should be used to disinfect the *Artemia* nauplii from viral, bacterial, fungal, microsporidian and other parasitic diseases and remove unhatched cysts from the *Artemia* nauplii. This will help maintain the larval-rearing tanks free from disease and organic material that serves as a nutrient source for disease organisms.

Harvest the hatched *Artemia* nauplii from the hatching tank into a 100 µm (250 mesh) net. Wash the nauplii thoroughly in running clean freshwater (preferably) or seawater. Place the washed nauplii into a 15–20 litre bucket. Add 10 litres of clean seawater. Add 125 ml of 50 percent hydrogen peroxide liquid and stir. Leave for 5 min and all the unhatched cysts and debris will form bubbles and float to the top of the bucket and the live nauplii will go to bottom. Use a 100 µm mesh net to scoop out all floating debris and throw it away. Pour the live nauplii into a 100 µm mesh net and wash thoroughly in running clean freshwater (preferably) or seawater.

Alternatively *Artemia* nauplii can be disinfected with a 20 ppm sodium hypochlorite solution, or better, chloramine-T at 60–100 ppm for 3 min, and washed with freshwater. However, these latter procedures will only disinfect the *Artemia* nauplii and not remove any unhatched eggs or cysts that may lead to contamination of the larval-rearing tanks. The *Artemia* nauplii are then ready to be fed live or frozen, or kept in a fridge at 4 °C at up to 5 million nauplii/litre for up to two days. They can then be fed when needed or placed into separate tanks for enrichment (for 3–12 h), or for on-growing for feeding to PL stages.

Frozen *Artemia* nauplii or adults should be stored in a separate, exclusive freezer. Basic hygienic protocols (SOPs) must be implemented at all times. For mysis (frozen) and early PL stages (live), only the nutritionally superior "umbrella" (just hatching) or instar I nauplii should be used as larval feed for the small-sized larval shrimp. However, for older PL, the *Artemia* metanauplii can be enriched with vitamins and minerals, probiotics and HUFA lipids to increase their nutritional value. Alternatively (and more economically), frozen, enriched adult *Artemia* biomass can be produced in-house or bought and fed (up to 12 times/d) from PL 5 until harvest.

After harvest the tanks used to hatch *Artemia* must be washed with detergent and water, and then disinfected using a sponge dipped in sodium hypochlorite solution (20 ppm active ingredient), rinsed with abundant treated (filtered and sterilized) water and washed again with a 10 percent solution of muriatic acid before sun-drying.

### 4.3.3 Artificial feeds

Many kinds of artificial or formulated feeds are available for use during larval rearing. These types of feeds generally do not pose the same health risks as live feeds because they can be maintained free from contamination. Generally they should not present any health-related difficulties as long as high quality feeds are selected, and they are stored correctly in cool, dry conditions, used promptly once the container is opened and not used excessively, as this can lead to water quality issues.

Artificial feeds include dried algae, liquid feeds, microencapsulated feeds, flakes and crumbled pellets, and mineral and vitamin supplements and enrichments. These are used in various sizes according to the stage of larval development and in various combinations depending upon hatchery preferences, water quality and nutritional requirements. However, they are usually used primarily as supplements to live feeds. Feeding regimes for artificial feeds will depend upon the quantity and frequency of live feed (algae and *Artemia*) offered, and a general guideline is shown in Tables 17 and 18.

## 4.4 IMPORTANT LARVAL DISEASES

### 4.4.1 Monodon baculovirus (MBV)

MBV is a major pathogen of shrimp that is enzootic to India and present in a large proportion of wild broodstock. Even though harmless during the initial stages, the virus may cause mortality in PL. However, mortality due to this virus is seldom reported in hatcheries or in farms, where its effects are seen more as slow growth if environmental conditions are sub-optimal.

MBV forms large, dark tetrahedral occlusion bodies (OBs) that occur mostly in the nuclei of the deep R and S cells of the hepatopancreas. They are eosinophilic (pink-staining) in the early stages and basophilic (dark-purple staining) in advanced stages, and occur in large numbers in a single nucleus. Early infection may be detected by the presence of hypertrophied nuclei with marginated chromatin and displaced nucleolus. The average size of the MBV nucleocapsid is 246 x 42 nm.

Severely infected PL may also exhibit reduced feeding and growth rates, increased levels of surface and gill fouling, and sometimes a white midgut line through the abdomen. However, definitive diagnosis is based on the histological demonstration of eosinophilic occlusion bodies in the nuclei of hepatopancreocytes. OBs may also be detected in fresh squash preparations of the hepatopancreas or in faeces stained with 0.05 percent aqueous malachite green. DNA probes for MBV and the PCR method of detection are also available.

The virus is transmitted by the ingestion of free virus and by cannibalism. It is also believed to be transmitted vertically (probable false vertically, attached to the outside of the eggs) from broodstock to offspring. Since the virus is easily found in the faeces of infected broodstock, it is probably transmitted to the eggs through faecal contamination during spawning. The virus may remain viable in the external environment for some time due to the protective nature of the polyhedral OB.

MBV can be controlled in hatcheries by avoiding contamination and by adopting strict disinfection regimes. All shrimp coming into the system should be quarantined and any infected animals should be removed and destroyed immediately. Water entering the facility must be thoroughly disinfected. All equipment and tanks should be thoroughly disinfected between cycles and equipment used in the spawning area should be segregated from the rest of the hatchery. Eggs should be separated from the spawner faeces and disinfected as detailed in Section 3.13. Exposure to 1 000 ppm calcium hypochlorite (400 ppm chlorine) for 24-h is necessary to inactivate MBV.

Often MBV prevalence in PL is related to stressful environmental conditions, and when a batch of PL tests positive for MBV, if water quality conditions can be improved, the batch can test negative on rechecking after a couple of days. Such procedures can save a lot of money if the hatcheries are educated as to their efficacy.

Finally, if SPF broodstock were to be made available and could be maintained in biosecure facilities, the current problems with MBV would be eliminated.

### 4.4.2 White Spot Syndrome Virus (WSSV)

Although WSSV does not have any direct adverse effects on the hatchery production of PL, many farmers will reject a batch of WSSV-positive PL. It has been conclusively shown that stocking PL with high levels of WSSV (as demonstrated by nested PCR) is more likely to result in a failed harvest then stocking uninfected PL. Production of WSSV-positive PL is thus a source of lost revenue for hatchery operators and is a high risk factor for the farmers.

WSSV enters the hatchery system either through water exchange, contaminated feeds or most commonly, through infected broodstock, of which greater than 60 percent are believed to be carriers in India. It is believed that in hatcheries the virus is vertically (probably false vertically on the outside of the eggs) transmitted from positive broodstock. Additionally WSSV in the hatchery can be transmitted via cannibalism of moribund shrimp larvae.

Detection is by histopathology by looking for hypertrophy of the cell nuclei of infected tissues, by DNA probes and through nested PCR analysis.

Prevention is the only cure, entailing testing and disposal of each infected broodstock; disinfection of water, tanks and equipment; and washing and disinfection of eggs/nauplii to prevent transmission of the virus from infected broodstock. Maintaining each batch of eggs, nauplii and larvae separate throughout the larval-rearing process can also reduce transmission of the virus within the hatchery.

Unfortunately apparently WSSV-free broodstock may still harbour the virus undetected until stressed, i.e. through spawning. Thus broodstock shrimp need to be tested following spawning (or some other stress) to ensure that they are really disease free, before continuing to culture larvae from the suspect shrimp. Of course development of SPF broodstock *P. monodon* would help eliminate this virus from the hatchery, but so far such animals are not commercially available.

### 4.4.3 Baculoviral midgut gland necrosis virus (BMNV)

Clinical signs reported from various Indian and Vietnamese hatcheries indicate the presence of BMNV in *P. monodon* hatcheries. BMNV is a rod-shaped, enveloped virus with a tail-like appendage and is 36 x 250 nm in size. It may be in the same family of viruses as WSSV and two insect viruses.

This virus is probably transmitted vertically or false vertically from the broodstock during spawning. It can appear in zoea or mysis, but it is most frequent in later PL stages. The first gross sign of infection is the pale, turbid appearance of the hepatopancreas ("white gut"). The epithelial cells of the hepatopancreatic tubules become necrotic with hypertrophied nuclei that contain large inclusion bodies and marginated chromatin. Severely affected PL may float inactively on the surface of water and display a white midgut line and white spots on the cephalothorax. Heavy mortality can result very rapidly.

Similarly for the other viruses affecting the hatchery, the only defense is prevention, including screening and discarding positive broodstock and proper disinfection of water and eggs/nauplii to prevent transmission from infected broodstock.

### 4.4.4 Vibriosis

Vibriosis has a high prevalence in all hatcheries in India and is caused by several species of bacteria belonging to the genus *Vibrio*. The disease appears with a variety of clinical signs such as necrosis of appendages; exuvial entrapment; reddening of the pleopods, pereiopods and gills; cessation of feeding; white intestine; excessive fouling; luminescence in the water and larval bodies and so on.

One of the most important species leading to vibriosis in India (and elsewhere) is *Vibrio harveyi*. On many occasions this species is present in such high numbers that high mortality results even after heavy doses of antibiotics. Due to the increasing resistance of many strains of *Vibrio* to a large number of antibiotics, a more comprehensive approach to their management in hatchery systems has to be adopted. This entails observing the strict biosecurity protocols detailed throughout this report, aimed at preventing the ingress and proliferation of pathogenic bacteria in the hatchery and reducing levels of stress. Additionally the use of probiotic bacteria that can out compete or inhibit the pathogenic bacteria can reduce incidences and/or the severity of vibriosis.

### 4.4.5 Larval mycosis

Larval mycosis, most often caused by *Lagenidium* and *Sirolpidium* spp., may be introduced into hatcheries through broodstock, live feeds and/or carrier hosts present in the seawater supply. Fungal spores can also arrive in the air and are ubiquitous in the hatchery. The fungal spores can also survive in seawater for long periods of time and readily attach to and encyst on the cuticle of the egg or the larvae or onto *Artemia* cysts. Prevention is thus almost impossible, although the regular filtration and disinfection procedures outlined in Sections 2.2 and 2.3 will help limit the problem. Once the spores start to grow, the most common method of treatment is to destroy the spores in the water by using either treflan (at 0.05–0.1 ppm up to daily) or benzalkonium chloride (BKC) (at 0.01–0.05 ppm daily). Treatment with UV light can also yield good results.

Microsporidians (as well as BMNV) have been implicated in the white-body and or white-gut disease found in Vietnamese hatcheries and may also be involved in diseases with similar gross clinical signs in Indian hatcheries.

### 4.4.6 Ciliate infestation

Stalked ciliate protozoans such as *Zoothamnium* and *Vorticella* spp. often grow in large numbers attached to the gills or appendages of the larvae, causing suffocation, cessation of feeding, moulting inhibition and death. Such infestations usually result from poor treatment of water, use of infected live feeds, inadequate water exchange and/or overfeeding. At the onset of infestation, treatment of larvae with 0.01–0.05 ppm of BKC may yield results. Later on during the PL stages problems generally become more severe due to the high organic loading of the larval tanks. In this case treatment with formalin (at 30 ppm for 1 h, with high aeration, followed by high water exchange) can usually eradicate the problem, provided the larvae are sufficiently healthy to withstand the treatment and to resume feeding and moulting.

### 4.4.7 Swollen hind gut (SHG)

Swollen hind gut syndrome has been a common problem in many Indian shrimp hatcheries since about 2002. Gross clinical signs are a bloated or swollen hind-gut area, with the posterior digestive tract appearing to convolute or meander through this distal portion of the tail. Signs only become apparent in the later PL stages from PL10 onwards. Although this problem rarely results in mortality, it has negative effects on digestion and excretion, and significantly reduces the value of the PL produced. This is because farmers suspect (although it is yet to be conclusively proven) that it results in digestive problems and differential growth rates during pond on-growing.

The cause(s) of this syndrome are not known, but may possibly include poor water quality (i.e. heavy metal toxicity), low quality/diseased nauplii, bacterial infection (Professor Donald Lightner's laboratory in the United States of America found only some Gram-positive bacteria in positive samples) and/or inferior quality dry feeds (use of liquid feeds appears to help avoid this problem). More research is urgently required to discover the causative agent(s) (if any) and remedial measures for this syndrome.

### 4.4.8 Diseases of unknown aetiology

Apart from the above mentioned specific diseases, there are reports from various hatcheries about varied health problems having characteristic signs, but for which no causative agent has yet been identified. These include: abrupt cessation of feeding and moulting, reduced growth rate, breakage of the body in the middle of the abdomen, appearance of an extra segment in the larval tail, differential expansion of the chromatophores on the underside of the tail leading to opaque tail muscle from PL9 onwards, and settlement of moribund larvae to the tank bottom without apparent infection. A comprehensive, multicentric research programme (with support from the industry) aimed at investigating the diseases of shrimp larvae in hatcheries is urgently required.

In case of high mortality due to disease, the infected larval-rearing tanks should be chlorinated as soon as possible (>50 ppm chlorine for 60 min) before being drained with care (into the waste-water treatment tanks) The larval-rearing tanks should then be suitably disinfected and thoroughly dried (preferably with direct exposure to sunlight) before they can be restocked with nauplii.

## 4.5 GENERAL ASSESSMENT OF LARVAL CONDITION

Routine assessments of shrimp health are an important component of good hatchery management to ensure that any potential problems are recognized early and solutions employed to rectify the underlying causes and thereby increase productivity.

A lack of knowledge regarding the importance of routine health monitoring, a lack of protocols for routine health monitoring, a lack of emphasis on disease prevention and the lack of a diagnostic and problem-solving approach are currently major impediments in Indian hatcheries. In Indian hatcheries, health monitoring and general checks on larval development, survival estimation, moulting and feeding regimes are conducted during the larval-rearing cycle. However, there is generally a weak monitoring system for water quality, larval health and survival rate, and routine bacteriology. Similarly organized recording systems are virtually absent, and PCR checking of broodstock and larvae is also very infrequent.

Assessment of larval condition is one of the most important activities carried out in the hatchery. The assessment is usually done in the morning, and decisions on water exchange, feeding and other management activities made so that action can be taken in the afternoon. All observations are recorded so that details are not forgotten and later problems can be referenced to historical records in each tank.

Samples of larvae and PL for routine checking should be taken in disposable plastic containers (paper cups or 300 ml plastic beakers) that are disposed of once used or in glass containers that can be disinfected. After the daily check is complete, the larvae or PL sampled should be discarded into a plastic container with sodium hypochlorite (20 ppm active ingredient) or another suitable disinfectant. Larvae and PL used in the daily checks must never be returned to the larval-rearing rooms or larval tanks.

The larvae in each tank should be inspected at least twice daily, preferably more frequently. Observations are made on the larval stage, health, activity, behaviour and abundance of feed and faeces in the water and the shrimp body. Records may also be taken of water quality parameters and the amount of food in the tank.

The same or a separate sample of larvae should also be taken to the laboratory for a more detailed microscopic examination. This will provide information on the stage, condition, feeding and digestion and the presence of any disease or physical deformity. Routine monitoring of the bacteriology of the larval-rearing tanks, at least at five stages (nauplius, zoea 2, mysis 2, PL1 and PL5) should also be conducted along with the routine larval health monitoring.

Samples should also be sent to a PCR laboratory once (two to three days before harvest) or twice (at nauplius and PL5) during the cycle for screening for viral pathogens (particularly WSSV and MBV).

*PL samples from hatcheries are being checked at the SIFT laboratory for diseases before purchasing. This practice reduces the risk of pathogens entering into ponds*

TABLE 19
**Diagnostic level descriptions adapted for use in shrimp hatchery systems**

| | |
|---|---|
| Level 1 | Observation of animal and environment. Examination based on gross features. |
| Level 2 | More detailed examination using light microscopy and squash mounts, with and without staining, and basic bacteriology. |
| Level 3 | Use of more complex methods such as molecular techniques and immunodiagnostics (e.g. PCR). |

The type of observations that are made can be categorized into three levels, based on the health assessment levels described in Table 19. They provide a simple and convenient separation based on the complexity of the techniques used.

### 4.5.1 Level 1 Health assessment observations

Level 1 observations are commonly employed in most hatcheries. Detailed examination of large numbers of larvae is not practical, and hatchery operators and technicians frequently use Level 1 techniques to get a preliminary feel of the health status of larvae and to prioritize more detailed examination. Level 1 observations are also frequently sufficient to make a decision about the fate of a hatchery tank or batch of larvae.

Selection of nauplii, for example, generally includes a decision based on phototactic response without the need for a more detailed microscopic examination. If a batch of nauplii shows poor phototaxis and weak swimming behavior, it should be rejected without the need for further examination. Likewise a severe case of white-body disease in early PL should be countered by destroying the larvae in that tank through chlorine disinfection before discharge to prevent transmission of the disease to other tanks of larvae in the facility.

Level 1 observations are based on simple visual features of the larvae and water condition that can be easily seen with the naked eye in a glass beaker of animals taken from the tank. Special attention is paid to the activity of the larvae, their swimming behaviour (according to the larval stage), water quality, presence of feed and faeces and later on, white-body disease, size disparity and homogeneity. These observation and the scoring system used are summarized in Table 20.

*Swimming activity*

The swimming activity of the larvae changes dramatically but characteristically through the larval cycle. Zoeal stages will swim rapidly and consistently forwards, usually in circles, filter feeding on phytoplankton. Mysis, by comparison, swim backwards with intermittent flicks of their tails, maintaining themselves in the water column and feeding visually on phyto- and zooplankton. PL again start to swim rapidly and consistently forward, searching for food while being maintained in the water column by strong aeration. Within these distinct modes of swimming, if >95 percent of the larvae are observed to be swimming actively, they are given a score of 10; if 70–95 percent are active, they are given a score of 5 and if < 70 percent are active, they are given a score of 0.

TABLE 20
**Summary of Level 1 assessments of larval health**

| Criteria | Score | Stage | Observation |
|---|---|---|---|
| Swimming activity<br>- Active (> 95%)<br>- Intermediate (70–95%)<br>- Weak (on bottom) (< 70%) | <br>10<br>5<br>0 | All stages | Daily (2–4x) observations |
| Phototaxism<br>- Positive (>95%)<br>- Intermediate (70–95%)<br>- Negative (< 70%) | <br>10<br>5<br>0 | Zoea | Daily (2–4x) observations |
| Faecal string (cord)<br>- Present (90–100%)<br>- Intermediate (70–90%)<br>- Absent (<70%) | <br>10<br>5<br>0 | Zoea | Daily (2–4x) observations |
| Luminescence<br>- Absent<br>- Present (<10%)<br>- Abundant (>10%) | <br>10<br>5<br>0 | Mysis | Night observation of the tank |
| White-body disease<br>- Absent<br>- Present (<10%)<br>- Abundant (>10%) | <br>10<br>5<br>0 | PL | Daily (2–4x) observation |
| Homogenous stage<br>- High (80–100%)<br>- Intermediate (70–80%)<br>- Low (< 70%) | <br>10<br>5<br>0 | All Stages | Daily (2–4x) observation |
| Intestinal contents<br>- Full (100%)<br>- Half full (50%)<br>- Empty (<20%) | <br>10<br>5<br>0 | Mysis | Daily (2–4x) observation |

### *Phototaxis*

Zoeal-stage larvae should retain a strong positive phototaxis and move towards light. To test this, a sample of larvae is placed in a translucent container (glass or beaker) next to a light source and the displacement of the animals is observed. The zoea should move strongly towards the light. Mysis and PL do not show such attraction to light. If 95 percent or more of the larvae move strongly towards the light, the larvae are good and given a 10; if 70–95 percent respond, they are acceptable and given a 5; and if less than 70 percent move towards the light, they are considered weak and given a score of 0.

### *Faecal string*

During the zoea l stages, when the zoea are feeding almost exclusively on algae, long faecal strings can be seen projecting from the anus and loose in the water column. When 90–100 percent of the larvae have these long, continuous strings all along the digestive tube, through their bodies and continuing outside, they are considered well fed and given a score of 10. When 70–90 percent have these strings or they are short or discontinuous, they are given a score of 5; and when <70 percent of the larvae have these strings, the larvae are not eating and are given a score of 0.

### *Luminescence*

This factor is observed directly in the larval-rearing tank in total darkness. Larval luminescence is generally due to the presence of luminescent bacteria such as *Vibrio harveyi*. There should be no luminescence in the tanks. If there is, it signifies high concentrations of potentially pathogenic vibrios and action must be taken, such as applying probiotics and/or changing water until the luminescence is gone. If the case is severe and treatment fails, the tank should be dumped quickly to prevent transfer of the infection to other tanks, as this problem is easily transferred and can result in mass mortalities. If no luminescence is observed, a score of 10 is given: if the observed

luminescence appears low (up to 10 percent of the population), the score is 5; and if above 10 percent of the population are luminescent, the score is zero.

***White-body disease***

There appear to be two forms of white-body disease (which may have separate causes and therefore be different diseases). The first form displays necrosis and whitening of the hepatopancreas and midgut, with white spots in the cephalothorax or a white line from the head to the tail. This disease causes rapid and heavy mortality. The second form shows a whitening of the tail where it bends in the 3rd abdominal segment, which gradually spreads throughout the entire body until causing death and the body to split into two pieces. This form shows lighter mortality. The water also appears to go reddish in colour, with white strands in it. If no white-body disease is observed, a score of 10 is given; if the observed disease appears low (up to 10 percent of the population), the score is 5; and if above 10 percent of the population have white bodies, the score is zero.

The exact causes of this disease(s) are unknown, but may be related to the presence of microsporidian parasites and/or viral disease such as baculoviral midgut gland necrosis virus (BMNV). Very occasionally, antibiotics are reported to work if treatment is applied quickly, but they are nearly always ineffective and the tank must be destroyed rapidly before disease can be transmitted to neighbouring tanks. Prevention through selection of disease-free broodstock, proper water disinfection and treatment, tank cleaning and the use of probiotics instead of antibiotics is recommended.

***Stage homogeneity (Size uniformity/size variation)***

This indicates the uniformity of larval stages in a tank. Most of the larvae should be within one moult stage of each other. Where there are many stages in a single tank, this indicates a problem (such as disease or poor water quality) needing attention. It should be noted that when larval shrimp moult, it is normal to see a decrease in the stage homogeneity, so the time at which the stage homogeneity is determined has to be taken into consideration. This is also true for PL when they are moulting. If 80 percent or more of the population is in the same stage, a score of 10 is given; if between 70–80 percent are at the same stage, the score is 5; and if less than 70 percent are in the same stage, the score is zero.

***Intestinal contents***

The intestinal contents (gut contents) can be observed in older larval stages. The intestine is visible as a dark line extending posteriorly from the hepatopancreas in the larva's head region that is easily observed in larvae held in a clear container such as a glass beaker. This is useful as a guide to larval feeding and feed availability. Most larvae should appear full and dark and if they do not, they are probably being underfed or are diseased and remedial action is indicated. If most of the larvae observed are full, a score of 10 is given; if half of the larvae have food in the intestine, a score of 5 is given; and if <20 percent of the larvae have food in the intestine, the score is zero.

### 4.5.2 Level 2 Health assessment observations

Level 2 observations are also frequently used in the decision-making process in shrimp hatchery management. All hatcheries should have a microscope that is used to make more detailed examinations of the condition of the shrimp larvae and to observe directly various health-related features. Level 2 observations are based on microscopic examination and squash mounts, if necessary, of a randomly taken sample of at least 20 larvae per tank (more for larger tanks). Special attention is paid to the state of the hepatopancreas, swollen hind gut (SHG) and intestinal contents, necrosis and deformity of limbs, fouling organisms and the presence of baculovirus in the faeces

TABLE 21
**Summary of Level 2 assessments of larval health**

| Criteria | Score | Stage | Observation |
|---|---|---|---|
| Hepatopancreas (lipid vacuoles) | | | |
| - High (>90%) | 10 | All stages | Daily (2–4x) observations |
| - Moderate (70–90%) | 5 | | |
| - Low (< 70%) | 0 | | |
| Intestinal content/Swollen hind gut | | | |
| - Full (>95%), (0% SHG) | 10 | All stages | Daily (2–4x) observations |
| - Moderate (70–95%), (1-10% SHG) | 5 | | |
| - Empty (< 70%), (>10% SHG) | 0 | | |
| Necrosis | | | |
| - Absent (0%) | 10 | All stages | Daily (2–4x) observations |
| - Moderate (<15%) | 5 | | |
| - Severe (>15%) | 0 | | |
| Deformities | | | |
| - Absent (0%) | 10 | All stages | Daily (2–4x) observations |
| - Moderate (<10%) | 5 | | |
| - Severe (>10%) | 0 | | |
| Epibionts | | | |
| - Absent (0%) | 10 | All stages | Daily (2–4x) observations |
| - Moderate (<15%) | 5 | | |
| - Severe (>15%) | 0 | | |
| "Bolitas" [1] | | | |
| - None | 10 | All stages | Daily (2–4x) observations |
| - 1 to 3 | 5 | | |
| - >3 | 0 | | |
| Baculovirus | | | |
| - Absent (0%) | 10 | Mysis | Daily (2–4x) observations |
| - Moderate (<10%) | 5 | | |
| - Severe (>10%) | 0 | | |

[1] Sloughed cells of hepatopancreas and intestine, expressed as number of "bolitas" in the digestive tract

or hepatopancreas of older larvae. These observations and the scoring system used are summarized in Table 21.

### *Condition of the hepatopancreas and gut*

The condition of the hepatopancreas and gut gives an indication of larval feeding and digestion. It is observed using a wet-mount of a sample of larvae on a microscope slide at a magnification of 40X. In healthy larvae showing active feeding and digestion, the hepatopancreas and midgut will be full of small, easily observed bubbles (digestive or "lipid" vacuoles) and strong peristalsis will be seen in the intestine. PL should not have a swollen hind gut (SHG) or a coiled posterior digestive tract. If the hepatopancreas appears empty or pale, without lipid vacuoles, then the larvae are either underfed and/or diseased and require treatment. If 90 percent or more of the animals sampled show abundant lipid vacuoles and/or a full gut without SHG, a score of 10 is given; if the sample shows 70–90 percent of individuals with lipid vacuoles and/or a moderately full gut or traces of SHG, a score of 5 is given; and if the gut is less than 70 percent full and/or the intestine is empty or there is >10 percent SHG, the score is zero.

### *Necrosis*

Necrosis of the larval body and limbs, which is an indication of cannibalism or possible bacterial infection, can be observed by light microscopy under low power. Necrosis should be absent. If found, it might signify underfeeding or poor water quality leading to increased bacterial concentrations, and improvements to the water quality should be made. If necrosis is absent, a score of 10 is given; where <15 percent of the animals show some necrosis, a score of 5 is given; and where >15 percent show necrosis, indicating a severe infection, a score of 0 is given.

***Deformities***

Deformities may indicate poor-quality nauplii, if in the early stages, and bacterial infections or mishandling and stress later on. Typically the fine setae on the limbs of the larvae and/or their rostra may appear bent, broken or missing; the tail may appear bent; or the gut may terminate before the anus. Typically no remedies exist for these problems (unless due to rough handling), and such deformed larvae will die. In severe cases it may be preferable to discard the whole tank as soon as possible to prevent infection of other tanks. Deformities should be monitored and if they are encountered frequently on many larvae, the water quality and disease status of the tank should be checked and rectified. Where deformities are absent, a score of 10 is given; if <10 percent have deformities, a score of 5 is given; and if >10 percent present deformities, a score of 0 is given.

***Epibiont fouling***

The larvae may become host to a range of fouling organisms ranging from bacteria and fungi through to protozoans of many species. These will typically attach to the exoskeleton on the head and body, and particularly around the gills of the larvae. Where the infections are slight, the next moult may remove the fouling without further problems, but in severe cases the fouling will persist or reoccur in the next stage, indicating poor water quality and necessitating action such as the application of 20–30 ppm formalin (with high aeration) for 1 h, followed by a large water exchange. Where fouling is absent, a score of 10 is given; if <15 percent have temporary or permanent fouling, a score of 5 is given; and if >15 percent are fouled continuously, a score of 0 is given.

***"Bolitas"***

"Bolitas" is the Spanish name given to a syndrome involving the detachment of epithelial cells from the intestine and hepatopancreas, which appear as small spheres within the digestive tract. It is believed to be caused by bacteria and can be fatal. Some success in preventing "bolitas" has been achieved by rapid stocking of the hatchery (within three to four days), use of probiotics, and good health and feeding management practices. If no bolitas are found a score of 10 is given; where 1–3 bolitas are present, a score of 5 is given; and when more than 3 are found a score of 0 is given.

***Baculovirus***

Baculoviruses can usually be detected in whole or squashed (stained with malachite green for Monodon baculovirus, MBV) preparations of hepatopancreas or faecal strands from larger-sized larvae, using a high-powered light microscope to spot the characteristic viral occlusion bodies (which, in the case of MBV, are dark coloured and tetrahedral). Apart from MBV, HPV and MGNV can and should also be assessed through stained hepatopancreas squashes. The expression of baculoviruses is often mediated by stress, and if seen, reductions in levels of stress (i.e. improving water quality) can often reduce prevalence and the associated problem of growth depression. Emphasis should be placed on prevention, which entails the selection of uninfected broodstock and disinfection of eggs and nauplii, together with proper disinfection and treatment of incoming seawater. Where baculoviruses are absent, a score of 10 is given; if <10 percent have baculovirus, a score of 5 is given; and if >10 percent are infected, a score of 0 is given.

All hatcheries should also routinely (preferably daily, or at least five times during each cycle) employ basic bacteriology to gain an understanding of the bacterial flora of the tanks and to identify possible pathogens when the larvae become weak or sick. If they do not have these facilities, samples should be sent to a competent laboratory for analysis, particularly if problems become evident. This information may then be used to make a decision on whether the tank should be discarded or treated (and how).

*The value of Level 1 and 2 scoring*

When all of these level 1 and 2 observations are made and recorded for each tank of larvae at each stage, an overall picture of larval health can be derived, with higher numbers relating to healthier larvae and vice versa. With experience it becomes easy to judge the overall health of each tank of larvae and to recommend courses of action to combat the problems encountered, depending on the scores obtained.

### 4.5.3 Level 3 Health assessment techniques

Level 3 techniques are becoming more commonly employed in shrimp hatcheries and in laboratories servicing such hatcheries. Polymerase chain reaction (PCR) methods are used for the screening of PL and broodstock for viral diseases, as are dot-blot and other immunodiagnostic tests.

PCR tests for at least WSSV (and preferably YHV and MBV) should be conducted on all broodstock held in the quarantine facilities. At least the heavily positive individuals should be destroyed immediately. PCR checks of PL (either once before sale or twice – including early PL) should be conducted using nested protocols for the same viruses to ensure only uninfected PL are sold to the farmers.

TABLE 22
**Summary of PL quality assessment using Level 3 procedures**

| Analysis | Observations | Qualitative determination | Score |
|---|---|---|---|
| PCR | WSSV | Negative | 10 |
| | YHV | Negative | 10 |
| | MBV | Negative | 10 |

TABLE 23
**Use of Level 1, 2 and 3 diagnostics in shrimp hatcheries**

| | |
|---|---|
| Level 1 | Examination of broodstock for general health condition, sex determination, staging of ovarian development, moult staging, removal of sick/moribund individuals. |
| | Selection of nauplii by phototactic response, zoea/mysis stage feeding by observation of faecal strands, larval activity, PL health, activity and behaviour, stress tests. |
| Level 2 | Examination of egg quality by microscope. Checking bacterial flora of normal or moribund animals. |
| | Microscopic examination of naupliar quality. Routine microscopic examination of larval condition and PL quality. Checking bacterial flora of rearing water and larvae. |
| Level 3 | Screening of broodstock by PCR. |
| | Screening of nauplii and PL by PCR. |

Level 3 assessments should be carried out on a statistically determined number of PL (usually 150 for a population >10 000) from each tank (in order to provide a 95 percent confidence level at 2 percent prevalence in the result) using PCR techniques for the detection of important viral pathogens. This testing must be done according to standard protocols by a competent health laboratory, following all the rules for sampling, preservation and transport of the samples. For a detailed discussion of sampling for disease detection, see OIE (2006).

The only acceptable result for any of these viral pathogens is a negative result (which scores 10 points – see Table 22), where both negative and positive controls have simultaneously given their corresponding expected results. All batches testing positive should be destroyed.

The various applications of the different diagnostic techniques in a shrimp hatchery are shown in Table 23.

## 4.6 QUALITY TESTING/SELECTION OF PL FOR STOCKING

Currently in India there is no universally accepted measure of PL quality and hence no baseline for comparison of different batches of PL. Different laboratories use different ways of reporting health status results so a harmonized approach to boost farmer confidence is urgently required.

The following protocol was developed by the Shrimp Biotechnology Business Unit of Mahidol University, Thailand to standardize PL quality assessment and help hatchery operators and pond farmers produce and select high quality *P. monodon* seed. The adoption of this objective protocol has resulted in a 30 percent premium being paid for PL that pass the assessment, particularly where this involves screening for WSSV. In India awareness programmes for the farmers to inform them about this quality testing system will also be required to get it universally adopted.