Technical Manual for Artificial Propagation of the Indonesian Catfish, *Pangasius djambal*
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 ISBN: 979-8186-92-3

Chapter V

Artificial Fertilization and Egg Incubation Techniques

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The artificial fertilization technique used for *P. djambal* is the dry method, i.e. the sperm is first spread over and mixed manually with collected ova.

To increase the fertilization rate it is recommended to divide collected ova in small batches of 100 – 200 g (100 – 200 mL) in plastic bowls. For fertilization, 5 mL of diluted sperm are poured over one 100-g (100 mL) batch of ova, then mixed delicately with a feather until the sperm is homogenously spread in the ova mass (Plate V.1). Spermatozoa activation is triggered by addition of freshwater. The ratio generally used is 1 volume of freshwater for 1 volume of ova. Freshwater should be added quickly in order to activate all spermatozoa at the same time. One minute of gentle stirring or mixing with a feather is recommended for good fertilization (Legendre *et al.,* 2000; Cacot *et al.,* 2002). Then eggs should be rinsed with clean freshwater to remove excess of milt before transferring them for incubation (Plate V.1).

Eggs of *P. djambal* are sensitive to mechanical shocks. As a consequence, shaking generally results in low hatching rates and increased proportion of deformed larvae. Egg manipulation should be done carefully to optimize hatching rates and numbers of normal larvae.

EGG INCUBATION TECHNIQUES

As is the case in *P. hypophthalmus*, eggs of *P. djambal* are sinking, spherical or slightly oval in shape and become sticky after contact with water. They adhere to each other or to any substrate via a sticky mucous coating covering their entire surface. Due to these characteristics, incubation techniques applied for *P. hypophthalmus* by fish farmers in Indonesia (Kristanto *et al.,* 1999) can also be used for *P. djambal* eggs.

Two of these incubation techniques have been tested and adapted for *P. djambal*; 1) incubation of eggs in monolayer in stagnant or running water and 2) incubation in running water funnels (MacDonald jars) after suppression of egg stickiness (Plate V.4).

As a general rule, it is preferable to incubate eggs in running water to remove continuously the waste materials produced by the eggs (NH_3, CO_2) and to maintain good quality and oxygenation of water. In most cases, running water also helps in limiting fungus development. Nevertheless, in Indonesia, many fish farmers commonly conduct incubation of fish eggs in stagnant water.

Incubation in stagnant water

Incubation in stagnant water is generally carried out in aquaria and does not need expensive equipment. This simple and cheap technology is the most widespread incubation system in Indonesia. However the drawback of this technique is the risk of water pollution by organic matter, particularly that accumulated from dead eggs. To limit this problem a limited quantity of egg should be incubated in each aquarium (maximum recommended of 100 eggs per litre). Therefore, for large scale production of fry, this technique requires a great number of aquaria, as well as a large space in the hatchery.

Structure preparation

In order to avoid a thermal shock, the aquaria must be filled with water well before receiving eggs to equilibrate temperature and increase dissolved oxygen concentration (by airstone).

Water used for egg incubation should be clear and treated with a disinfectant such as formalin to control fungus development (*Saprolegnia* sp.). In order to avoid toxic effects of the drug on the eggs, this treatment should be applied 12 h before placing eggs in incubation. A dosage of 10 to 15 $mL.m^{-3}$ formalin inhibits fungus development and disinfects incubating water without any risk for the eggs and young larvae.

Egg incubation

Homogeneous egg distribution in a monolayer at the bottom of the tank is decisive for the success of incubation. This allows each egg to be in water of good quality. In this way, healthy eggs are not affected by dead eggs starting to decompose.

After fertilization and rinsing off excess of milt, the aeration is stopped in the aquarium and eggs can be delicately spread out with a feather on the water surface. (Plate V.2). It is then recommended to mix eggs and water gently to obtain a homogeneous distribution of eggs in the aquarium. As eggs of *P. djambal* are sinking, the previous homogeneous distribution will allow the eggs to reach the bottom of aquarium in a monolayer (Plate V.3).

After some minutes and eggs have stuck to the glass, the aeration can be progressively opened again without disturbing the incubating eggs.

Incubation in running water funnel (MacDonald jar)

The MacDonald jars used at the RIFA and JFADC stations are funnel type with a spherical bottom made of fiberglass (Plate V.2). Farmers also use other materials such as glass, concrete, plastic and non-corrosive steel.

The MacDonald jar principle consists in keeping eggs in motion via a water inflow through a PVC pipe fixed into the funnel and reaching right down to

the bottom (Woynarovich and Horvath, 1980). It is generally connected to a clean gravity-flow water or a recycling water system, so this technique presents the advantages of occupying limited space in the hatchery and reducing fungus development (*Saprolegnia* sp.) on eggs during incubation.

After hatching, this system also facilitates sorting newly hatched larvae from white (dead) eggs and egg shells.

Removing the stickiness of eggs

After fertilization and before pouring them in the MacDonald jar, eggs have to undergo a treatment with clay to suppress their stickiness (Plate V.4). Actually, after mixing eggs and clay together, small particles of clay adhere to the egg sticky layer, covering their entire surface and preventing them adhering to each other or to any substrate. The suppression of adhesiveness allows keeping the eggs in movement in the water flow during the entire incubation time.

Preparation

The clay suspension is made of 1 kg of red clay (Latosol) in 2 litres of water. The procedure is as follows:

- red clay is cleaned of its impurity (leaf, pebbles, etc.);
- it is then mix progressively with boiling water (ratio of 1 kg for 2 L). Boiling water is necessary to kill microorganisms and parasites. After mixing, the preparation could be boiled again to ensure its sterilization;
- after cooling, the solution is sieved through a 700 µm mesh;
- the sieved mixture is kept in a plastic bucket fitted with strong aeration to obtain homogeneous mixing;
- the preparation is ready to use. If in excess after a given reproductive trial, the remaining clay suspension can be preserved frozen until another induced breeding. The clay suspension should be frozen in small volume to allow thawing according to the quantity needed.

Suppressing the stickiness

After mixing of sperm and ova for artificial fertilization (see above) excess milt should be drained off and replaced by clay suspension following the procedure below (Plate V.4):

- pour about 100 mL of clay suspension on 200 g of eggs;
- delicately mix the eggs and the clay using a feather until they do not adhere to each other, i.e. clay already covers all the sticky mucous;
- then the mixture is transferred into a net to remove the excess clay;
- after rinsing until clean water is obtained, eggs are transferred again to a plastic bowl filled with water;
- eggs are then ready to be placed in the MacDonald incubators.

Structure preparation

When MacDonald jars are connected to a recycling water system:

- a special procedure for first using recycling water system should be followed as described in Chapter VII (see "preparation of rearing structure"). In all cases, incubators must be filled with clean water and run long enough before receiving eggs for equilibrating temperature and reaching maximal level of dissolved oxygen;
- a preventive treatment of formalin at a concentration of 10 to 15 $mL.m^{-3}$ is also recommended in order to disinfect the incubation water.

When incubators are connected to gravity-flow water, water should be:

- free of plankton and waste;
- well oxygenated;
- at stable and adequate temperature (27 – 30°C);
- distributed at a constant flow.

Egg incubation

After suppression of their stickiness, a maximum of 200 g of eggs can be transferred delicately into each jar (capacity of 20 L). Before pouring eggs into their incubator, water flow should be stopped for a while in order to avoid loss of eggs through the water outlet. After eggs have sunk to the bottom, the water inlet can be opened slowly and adjusted for continuously keeping eggs in motion. During incubation, the adjustment of the rate of flow and the centering of PVC inlet pipe are essential for optimizing hatching rates (Plate V.5):

- insufficient outflow or bad centering of the inlet pipe may create a motionless mass of egg which could not be well oxygenated. This could result in mortality of a significant proportion of embryos due to anoxia, with dead eggs turning white;
- too strong outflow shakes up eggs excessively and risks impairing embryo development resulting in increased proportion of deformed embryos and larvae;
- good adjustment of water flow and centering of inlet pipe result in all the eggs getting slow and regular wave motion.

EMBRYO DEVELOPMENT AND HATCHING KINETIC

Embryo development

As the process of embryo development is not the main topic of the present work, we will limit this presentation to some important stages allowing

farmers to recognize them and correctly evaluate egg quality. The duration of incubation (from fertilization to hatching) depends upon water temperature, it decreases as temperature increases (Legendre *et al.*, 1996). The following description gives an example of development time for a mean incubation temperature of 29°C. Corresponding egg development illustrations are given in Plate V.6.

After contact with water, eggs are subjected to a rapid hydration leading to the formation of the perivitelline space. The egg mucus coat also swells in contact with water and becomes adhesive. At this stage, whether fertilized or not, the swollen eggs have a yellowish color and the animal pole is marked as a reddish-brown cap. However, fertilized eggs soon start to develop and the first cleavage (stage two-cells) becomes clearly visible 25 – 30 minutes after fertilization, followed by 4, 8, 16 and 32-cell stages (1.5 to 2 hours after fertilization). From the 32-cell stage, the egg is in morula stage during about 60 minutes and then cells become progressively smaller until the blastula stage (3 to 4 hours after fertilization). Shortly thereafter, the gastrula stage starts, the cell division progresses and cells progressively cover the yolk mass. The last step of the gastrula stage occurs about 12 hours after fertilization and is characterized by the closing of the blastopore.

Thereafter, i.e. 15 to 18 hours after fertilization the embryo appears in the form of a half ring with the head and the tail buds present at the two ends. Afterwards the first segments of the body become rapidly visible while the tail bud starts to grow longitudinally. The cardio-vascular system becomes functional and the embryo starts to twitch its tail occasionally. The first pigmented cells (melanophores) become clearly visible after 23 hours from fertilization and the movements of the embryo become more and more vigorous before hatching.

Differences between fertilized and unfertilized eggs

As noted earlier, once exposed to water, unfertilized eggs start the swelling process and their animal pole is marked by a reddish capsule as is the case for fertilized eggs. Moreover, incubating unfertilized eggs can remain translucent for several hours and can not be easily distinguished from fertilized eggs with the naked eye. It is only after 8 to 14 hours of incubation that most unfertilized eggs become opaque and whitish.

When observed with a low power stereomicroscope (magnification x 25), unfertilized eggs of *P. djambal* do not show any cell divisions. For an accurate and easier evaluation of fertilization rate, it is recommended to observe eggs between the “4-cell” and the morula stages; i.e. from about 30 min to 2 h after fertilization (Plate V.6).Estimation of fertilization rates should be done during these early development stages, because at following stages, i.e.

from late morula to early gastrula (between about 2 to 5 h from fertilization), fertilized eggs again become more difficult to distinguish from unfertilized ones.

Determining fertilization rate allows farmers to rapidly evaluate expected hatching rate from a given spawn. Actually, a low fertilization rate (< 30 – 50%) is generally indicative of poor ova quality when the ova are properly obtained respecting all rules for sperm conservation, ova fertilization and egg incubation. Due to abnormality of embryonic development and increased embryo mortality, low fertilization rates resulting from bad quality of ova generally lead to even lower hatching rates and high proportions of deformed larvae. In such case, it may be more advisable for farmers to clean off eggs and induce new broodfish in order to obtain eggs and larvae of better quality.

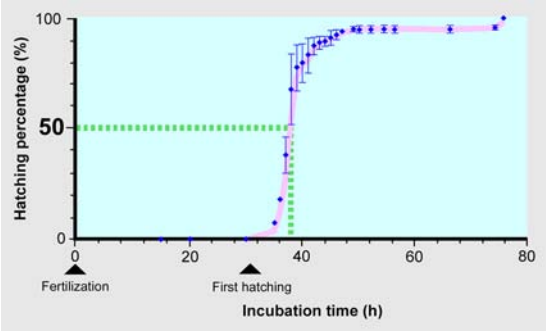
Duration of incubation

In *P. djambal*, as in other fish species, the duration of egg incubation is strongly dependent upon water temperature. Hatching time could be reached faster in warm water and retarded in cold water. At a water temperature of 29 – 30°C, larvae started to hatch by breaking out of the egg shell, about 33 to 35 h after fertilization (Figure V.1). Fifty percent of larvae were already hatched after 37 – 38 h; i.e. 2 – 3 hours after the beginning of hatching.

Larvae do not hatch in synchronization and the difference between the first hatching and the last hatching could be up to 40 h (Figure V.1). However, such long delay generally corresponds to hatching of deformed larvae and 9 to 10 h after the first hatching, more than 90% of larvae had already broken out of the eggshell.

In every case, the incubation temperature should be adapted to the species requirement to obtain the optimal hatching rate. For *P. djambal*, a water temperature of 27 to 30°C was shown to fall within a suitable range.

Figure V.1.
Hatching kinetics of *P. djambal* eggs incubated at a water temperature of 29 – 30°C.



HANDLING AND STOCKING NEWLY HATCHED LARVAE

After hatching, water in incubating structures is usually polluted by decomposing dead eggs and hatching wastes. Water begins to turn unclear, indicating that the environment could become toxic and dangerous for newly-hatched larvae.

To avoid a risky situation, it is necessary to quickly transfer larvae into "temporary" structures containing clean stagnant and aerated water. This could be aquaria (60 to 120 L) or small tanks. At this stage, oxygenated stagnant water is recommended because larvae cannot swim and a small current could be enough to squash them on the outlet net (Plate V.7).

This transfer could start as soon as possible after most larvae have hatched (e.g. 40 h after fertilization or 5 h after first hatching at 29 – 30°C).

"Temporary" structures must be ready long time enough before receiving the larvae to maximize dissolved oxygen concentration and equilibrate water temperature. It is important avoiding thermal shock or stress to larvae.

A few hours after hatching, larval behavior changes and normal larvae become more active and photosensitive (attracted by light). From this moment, it is relatively easy to concentrate normal larvae at one side of the aquarium or tank, and to separate them from those deformed and unable to swim. This operation should be repeated until no more normal larvae gather up. (Plates V.3 and V.7). As the first hatching occurred about 40 h before the last hatching, larvae obtained from a same group of eggs are not exactly at the same stage of development.

This step is important for beginning larval rearing in good condition because most abnormal larvae are doomed to die before 2 – 3 days of age and decomposing bodies can pollute the rearing environment, favoring bacteria and fungus development.

Technical guidelines

Before use, all the material should be cleaned, disinfected and dried.

- After fertilization and stocking eggs in incubation system, i.e. around 40 h (at 29 – 30°C) before collecting larvae, fill up "temporary" containers with clean freshwater and implement a strong aeration. This period is long enough to equilibrate oxygen and temperature;
- to avoid stress, before moving newly hatched larvae into the "temporary" containers, reduce aeration and control temperature. Water temperature

should be in the recommended range (see Chapter VI, Table VI.2) and close to that of incubation water (± 1°C);

- fill up a plastic bowl with clean water from the "temporary" tank and implement a gentle aeration, then catch larvae with care from the incubation structure with an adapted plankton net (80 µm) and transfer them slowly into the bowl (Plate V.7);
- when enough larvae have been caught, slowly empty out the bowl into a "temporary" container (Plate V.7);
- in "temporary" tanks, clean a part of the bottom by siphoning to remove white eggs and abnormal larvae. One to two hours after moving larvae, give some light on this clean bottom. Wait for about half an hour to concentrate normal larvae in the beam of light (Plate V.3 to V.7);
- while waiting for larvae aggregation, fill up a plastic bucket with clean water from the future rearing tank and implement a gentle aeration. Water temperature has to be in the recommended range of 27 – 31°C with no more than 1°C difference from water of the "temporary" containers. Siphon larvae slowly with a plastic pipe into the bucket. This operation has to be repeated until all normal larvae have been siphoned (Plate V.7);
- before transferring larvae in each rearing tank, their number has to be determined by counting or at least accurately estimated in order to adjust feeding and water renewal during larval rearing (Plate V.7).

EQUIPMENT AND TOOLS

Artificial fertilization

1. Clean and dry plastic bowl for dividing collected ova.
2. Clean and dry syringe of 10 – 30 mL capacity for evaluating volume of sperm collected.
3. Measuring cup or bowl for freshwater.
4. Chicken feather for mixing eggs, sperm and freshwater.
5. Clean freshwater for rinsing eggs and eliminating excess sperm after fertilization.

Incubation in stagnant water

1. Aquarium 60 x 50 x 40 cm.
2. Air blower with airstone in each aquarium.
3. Plastic bucket for filling aquaria.
4. Tank for storing clean water.
5. Chicken feather for dispersing eggs into aquaria.

Incubation in running water funnel

1. Recycling water system with bio-filter and mechanical filter or clean gravity water.
2. MacDonald jars installed as explained in Plate V.5.

Removing the stickiness of eggs

3. Sterilized clay suspension (1 kg for 2 L of water).
4. Plastic bowl and feather for mixing.
5. Small net and clean freshwater for removing excess clay.
6. Measuring cup or bowl for pouring the same quantity of eggs in each jar.

Handling and stocking newly hatched larvae

1. Aquaria with airstone and clean water.
2. Plankton net (80 µm mesh size) for catching larvae.
3. Plastic bowl for transferring larvae to the aquaria.
4. Flashlight for concentrating normal larvae in the beam of light.
5. Plastic pipe for cleaning the bottom of white eggs and deformed larvae and then siphoning normal larvae.
6. Plastic bucket for transferring normal larvae to their rearing structures.

Estimation of fertilization rate

1. A low power stereomicroscope (magnification x 25).

Water quality control (recommended)

1. Oxygen kit or oxygen measuring device.
2. Thermometer.

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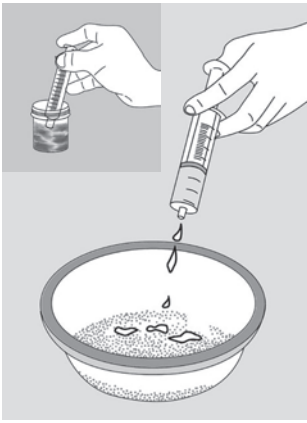
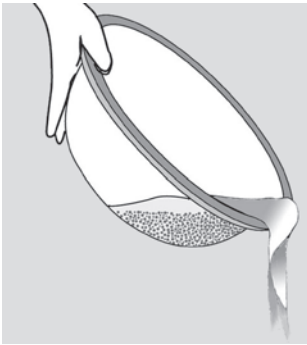
To optimize fertilization rate, divide ova into small batches of 100 – 200 g (100 – 200 mL).



- Pour 5 mL of diluted sperm for 100 g (100 mL) of ova.
- Mix delicately until sperm is well spread.

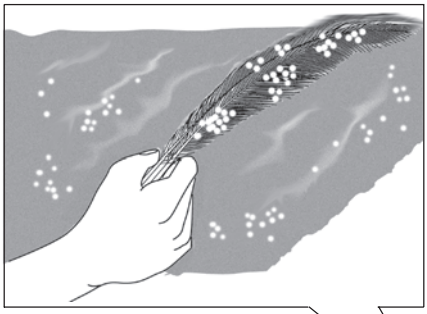
- Activate spermatozoa by addition of 1 volume of freshwater for 1 volume of ova.
- Freshwater has to be poured quickly to activate all spermatozoa at the same time.



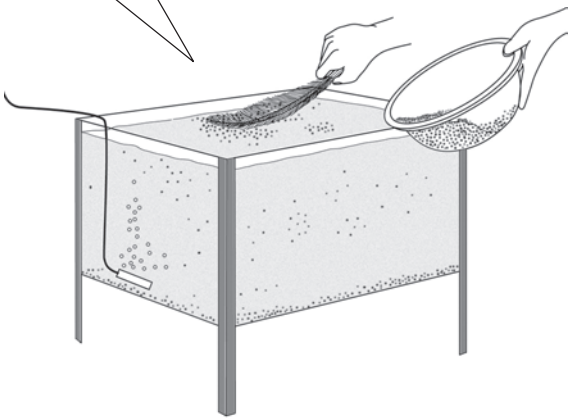
- Mix slowly for 1 minute.
- Rinse with freshwater to remove excess milt before transferring eggs to incubators.

Plate V.1.

Procedure of ova fertilization.



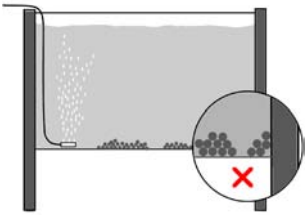
- After fertilization, handle eggs delicately using a feather.
- Disperse up eggs into aquarium.
- Mix eggs and water to obtain a good distribution of eggs over the entire water column. Then they will sink with an homogeneous distribution to the bottom and adhere to the glass surface.

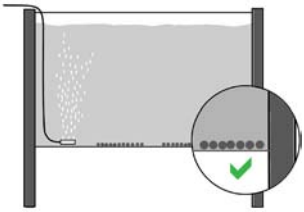


- After suppressing their stickiness (Plate V.4), transfer rinsed eggs into a plastic bowl filled with clean water and slowly pour them into the MacDonald incubator.

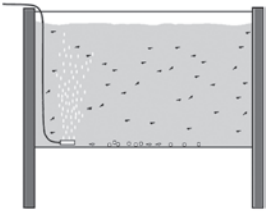
Plate V.2.

Transfer of the fertilized ova to the incubation structures.

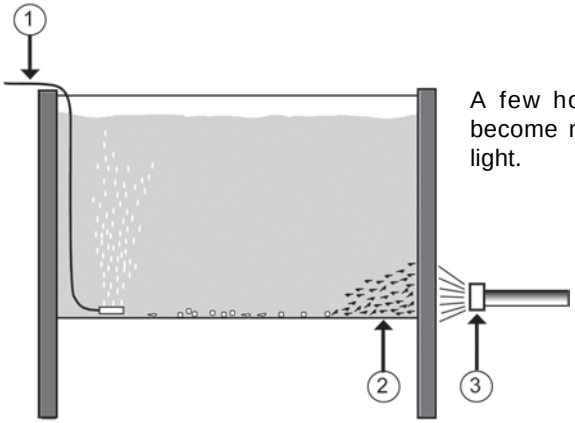




Distribution in a monolayer on the bottom allows each egg to get good water quality and improves hatching rate.



Larvae start to hatch after 33 to 35 h of incubation at 29 – 30°C and after a while swim throughout the entire column of water.



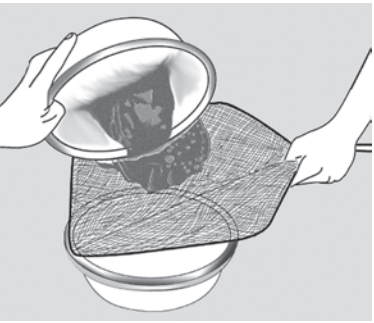
A few hours after hatching, larvae become more active and attracted by light.

1. Airstone.
2. Normal larvae gather in the beam of light.
3. Flashlight.

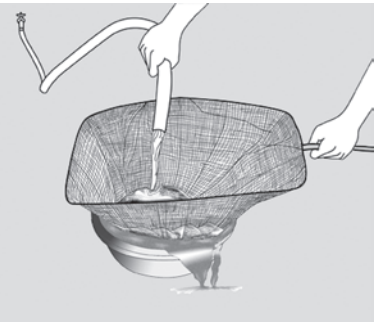
Plate V.3.

Incubation in stagnant water.

- Pour 100 mL clay solution on 200 g of eggs.
- Mix delicately with a feather until eggs do not adhere to each other.



- Delicately transfer the eggs in a small net to remove the excess clay.

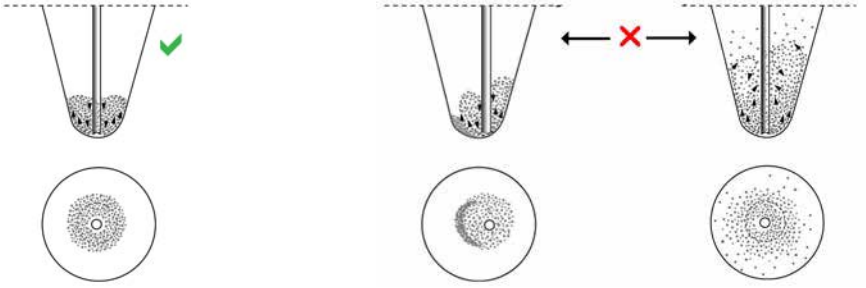


- Rinse with a slow rate of flow until clean water is obtained.

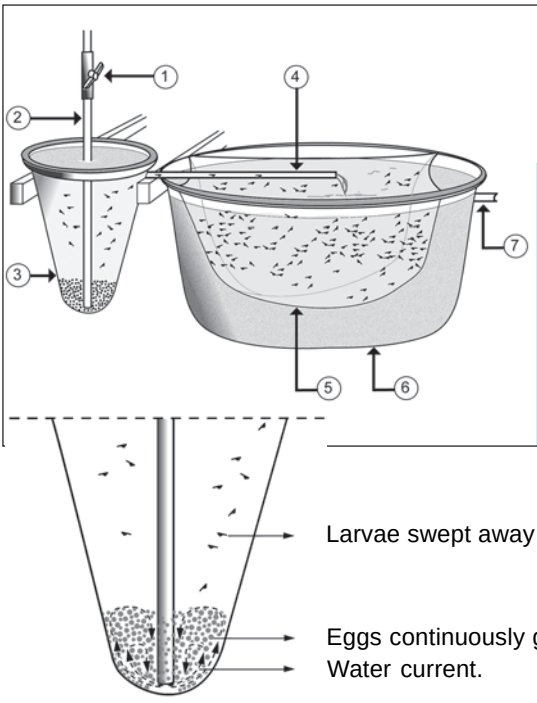
- Transfer eggs into a plastic bowl filled with clean water.
- Eggs are ready to be incubated in MacDonald jars.

Plate V.4.

Procedure for suppressing stickiness of eggs.



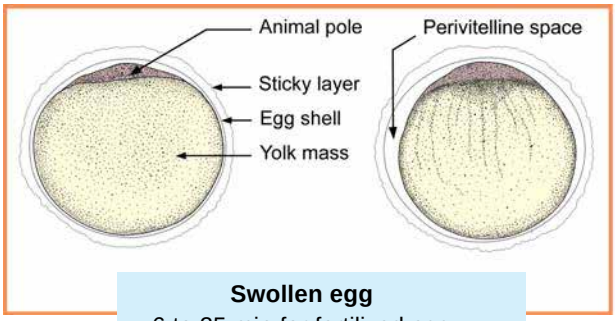
1. Adjusting water flow allows the surface of the egg mass to get slow wave motion.
2. Bad centering of the inlet pipe does not allow setting all the eggs in motion.
3. Strong water flow shakes up eggs and risks increasing embryos abnormality.



1. Outflow adjustment.
2. PVC pipe.
3. Eggs in motion.
4. Over flow.
5. Small size net receiving newly hatched larvae.
6. Fiber tank.
7. Over flow.

Plate V.5.

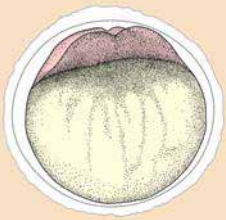
Incubation in MacDonald jar.



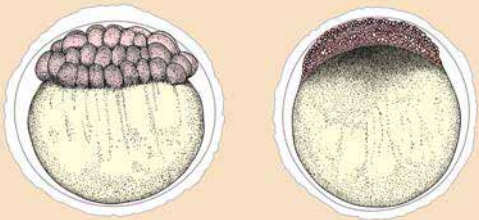
Swollen egg
- 6 to 25 min for fertilized egg.
- Such aspect could last for more than 8 h for unfertilized egg.

2-cell stage
25 to 40 min

Best stages for evaluating the fertilization rate



4-cell
30 to 55 min



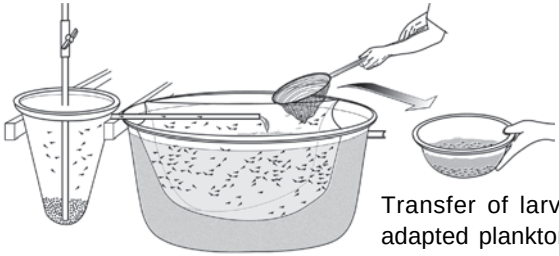
Morula
1 to 2 h

Gastrula
(middle time)
about 7 h

Closing of the Blastopore
12 to 18 h

Plate V.6.

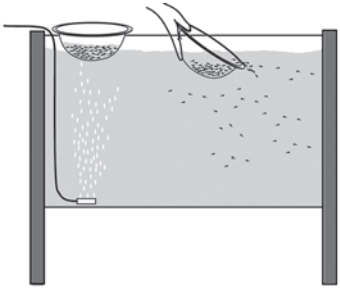
Some early stages of *P. djambal* embryo development.

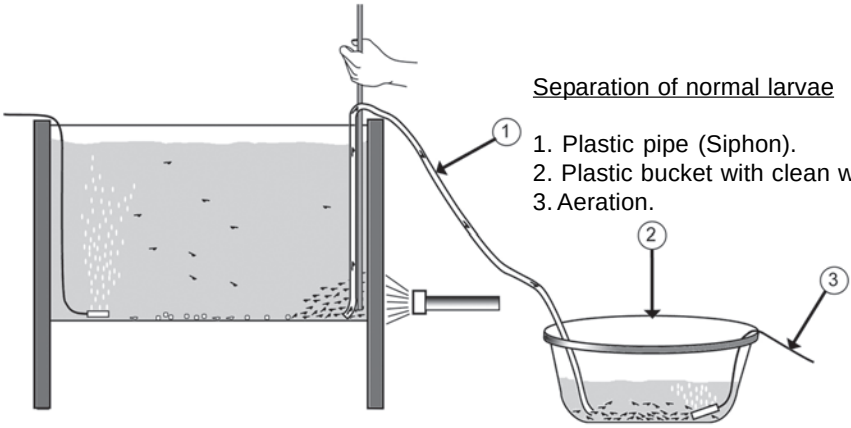


Transfer of larvae with adapted plankton net.

To avoid risks, it is necessary to transfer larvae soon after hatching into “temporary” structures containing clean aerated water.

Equilibrating temperature is strongly recommended, before slowly emptying out the bowl.





Separation of normal larvae

1. Plastic pipe (Siphon).
2. Plastic bucket with clean water.
3. Aeration.

Before being transferred to their rearing structures, larvae should be counted for better management of feed distribution and water quality.

Plate V.7.

Harvest of newly hatched larvae.

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Jakarta, 2003

DEPARTEMEN KELAUTAN DAN PERIKANAN

Title: Technical Manual For Artificial Propagation Of The Indonesian Catfish, *Pangasius djambal*

Editors: Jacques Slembrouck, Oman Komarudin, Maskur, Marc Legendre

Pictures: Jacques Slembrouck
Cover, layout and illustrations: Bambang Dwisusilo

ISBN: 979-8186-92-3